

SALIVARY GLAND DISEASE: A ROLE FOR BK VIRUS

by:

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ABSTRACT

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(Upon the direction of Jennifer Webster-Cyriaque)

Salivary gland disorders (SGD) cause significant morbidity but remain an enigma. SGD includes autoimmune disorder Sjogren's syndrome (SS), and a related HIV-associated salivary gland disease (HIV-SGD), that is AIDS defining in children and increasing in the adult population. Each of these maladies is characterized by loss of exocrine function resulting from chronic immune attack directed against the salivary glands with CD4+ (SS) or CD8+ (HIV-SGD) lymphocytic infiltrates within the gland tissue. The affected individuals are disfigured by facial asymmetry and 1-10% develop malignant lymphoproliferative disease. Further, the associated xerostomia instigates caries, mucosal pathology and periodontal disease. Despite progress made toward understanding aspects of SS, critical gaps remain in understanding the pathogenesis of SGD. The long term goal of this project is to make critical strides toward understanding the etiology of SGD in order to go beyond the ineffective palliative treatment that is currently the standard of care.

The central hypothesis of this work is that viral pathogenesis is essential to the development of salivary gland disease. Results show for the first time that polyomavirus,

BKV is associated with salivary gland disorders. BKV DNA and its gene products were consistently detected in HIV-SGD salivary gland biopsies and in a subset of SS patient. BKV was also detected in the peripheral blood and shed in oral fluids from HIV-SGD patients and not in control subjects. To confirm the in vivo findings an in vitro model was created whereby parotid and submandibular salivary gland cells were productively infected with BKV demonstrating each part of the viral life cycle. Salivary gland tropism was confirmed and the BKV receptor on salivary gland cells was defined. BKV transmission and pathogenesis is not well understood. Importantly, these studies suggest that BKV transmission may occur via the oral route.

Differential gene expression studies were also performed on HIV-SGD salivary gland biopsies. Among the many modulated transcripts was a novel non-coding RNA, MALAT-1, that was highly up-regulated in the disease. Further investigation of this transcript revealed that BKV infection played an important role in its regulation and we report for the first time a mechanism for MALAT-1 regulation.

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TABLE OF CONTENTS

LIST OF TABLES.....	ix
LIST OF FIGURES.....	x
ABBREVIATIONS.....	xii
CHAPTER 1: INTRODUCTION	
Salivary Gland Diseases.....	2
HIV-associated Salivary Gland Disease (HIV-SGD).....	2
Diffuse infiltrative lymphocytic syndrome (DILS).....	5
Sjogren’s Syndrome.....	6
SGD’s: infectious etiologic agents?	9
Viruses and salivary gland disorders.....	10
Differentially expressed genes in salivary gland disorders.....	14
Polyomaviruses.....	15
BKV.....	19
Virion Structure.....	19
Genome Organization.....	19
Life Cycle.....	23

Route of transmission and seroconversion.....	24
Pathogenesis, dissemination and persistence.....	25
BKV-associated diseases.....	26
BKV in Immune suppression.....	26
BKV-associated nephropathy (BKN).....	26
Hemorrhagic cystitis.....	27
Human Immunodeficiency Virus.....	28
Autoimmune disease.....	29
BKV and Cancer.....	30
Objectives.....	31
References.....	34

CHAPTER 2: HIV-SALIVARY GLAND DISEASE IS ASSOCIATED WITH BK VIRUS

Abstract.....	49
Introduction.....	50
Materials and Methods.....	52
Results.....	55
Discussion.....	65
References.....	69

CHAPTER 3: BK VIRUS DETECTED IN SALIVARY GLANDS OF SJOGREN SYNDROME

Abstract.....	73
Introduction.....	74
Materials and Methods.....	75

Results.....	78
Discussion.....	84
References.....	87
CHAPTER 4: BK VIRUS UP-REGULATES A NOVEL NON-CODING RNA ASSOCIATED WITH METASTASIS, MALAT-1	
Abstract.....	90
Introduction.....	91
Materials and Methods.....	94
Results.....	100
Discussion.....	110
References.....	115
CHAPTER 5: BK VIRUS HAS TROPISM FOR HUMAN SALIVARY GLAND CELLS IN-VITRO: IMPLICATIONS FOR TRANSMISSION	
Abstract.....	118
Introduction.....	119
Materials and Methods.....	121
Results.....	127
Discussion.....	144
References.....	148
CHAPTER6: GENERAL CONCLUSION	
References.....	161

LIST OF TABLES

CHAPTER 1

Table 1.1 Comparison of salivary gland disorders: HIV-associated

Salivary Gland Disease vs. Sjogren's Syndrome.....8

Table 1.2 Viruses detected in the salivary gland.....13

Table 1.3 The Polyomaviruses.....17

CHAPTER 4

Table 4. Genes up-regulated in HIV-SGD using suppressive
subtractive hybridization.....93

LIST OF FIGURES

CHAPTER 1

Figure 1.1 Virion structure and genomic organization of BK virus.....	21
---	----

CHAPTER 2

Figure 2.1. Detection of Polyomavirus DNA in HIV-SGD and not Herpesviral DNA in HIV SGD.....	56
--	----

Figure 2.2. Detection of BK virus in HIV-SGD.....	59
---	----

Figure 2.3. Detection of BKV gene expression in HIV-SGD.....	62
--	----

Figure 2.4. Detection of BKV viremia and shedding in oral fluids of HIV-SGD.....	64
--	----

Figure 2.5. Proposed Role for BKV as an infectious agent in the development of HIV-SGD.....	68
---	----

CHAPTER 3

Figure 3.1. BKV DNA detected in a subset of SS patients.....	79
--	----

Figure 3.2. Sequence analysis confirmed the detection of BKV DNA in SS.....	80
---	----

Figure 3.3. BKV gene expression in a subset of SS.....	82
--	----

Figure 3.4. Up-regulation of a novel non-coding RNA, MALAT-1 in a subset of SS.....	84
---	----

CHAPTER 4

Figure 4.1. Up-regulation of MALAT-1 in salivary gland disease.....	103
---	-----

Figure 4.2. BKV detected in salivary gland biopsies of HIV-SGD patients.....	105
--	-----

Figure 4.3. BKV up-regulates MALAT-1 transcript in vitro.....	108
---	-----

Figure 4.4. BKV infection up-regulates MALAT-1 promoter activity.....	111
---	-----

CHAPTER 5

Figure 5.1. BKV DNA detection in oral fluids.....	128
Figure 5.2. Detection of BK virions in salivary gland cell lines infected with BKV.....	130
Figure 5.3. BKV replicates in human salivary gland cells.....	132
Figure 5.4. BKV protein expressed in salivary gland cells.....	134
Figure 5.5. BK virions detected within salivary gland cells.....	135
Figure 5.6. Encapsidated BK virions released from human salivary gland cells.....	137
Figure 5.7. Infectious virus released from HSG cells	139
Figure 5.8. Inhibition of BKV infection in salivary gland cell lines via gangliosides.....	142

CHAPTER 6

Figure 6. Proposed model of BKV pathogenesis in a patient with salivary gland disease.....	160
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ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
ANA	Antinuclear antibodies
APV	Avian polyomavirus
ART	Antiretroviral treatment
BFDV	Budgerigar fledgling disease virus
BKN	BK nephropathy
BLC	Benign Lymphoepithelial Cysts
BMT	Bone marrow transplant
bp	Base pair
BSA	Bovine serum albumin
ByPV	Bovine polyomavirus
C	Catalytic
cDNA	complementary DNA
CMV	Cytomegalovirus
CsCl	Cesium chloride
CSF	Cerebral spinal fluid
DILS	Diffuse infiltrative lymphocytic syndrome
DMEM	Dulbecco's minimal essential medium
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
EMC	Encephalomyocarditis
EMSA	Electrophoretic mobility shift assay

ER	Endoplasmic reticulum
FBS	Fetal bovine serum
Go	Growth zero
h	Hour
HAART	Highly active antiretroviral therapy
HaPV	Hamster papovavirus
HC	Hemorrhagic cystitis
HHV	Human herpesvirus
HHV-6	Human herpes virus 6
HHV-7	Human herpes virus 7
HIV	Human Immunodeficiency virus
HLP	Hairy leukoplakia
HPTE	Human proximal tubule epithelial
HPV	Human papillomavirus
HSG	Submandibular salivary gland cells
HSY	Parotid gland salivary gland cells
HTLV	Human T lymphotropic virus
IFITM1	IFN- induced transmembrane proteins
IHC	Immunohistochemical
IRD	Immune reconstitution disease
ISGF3G	Interferon- stimulated transcription factor 3γ
JCV	JC virus
kb	Kilobase

kD	kilodalton
KPV	K Papovavirus
KPV	Kilham Virus
KS	Kaposi's sarcoma
KS	Kaposi's sarcoma
KSHV	Kaposi's sarcoma herpes virus
LPV	Lymphotropic papovavirus
LT Ag	Large T antigen
MALAT -1	metastasis- associated lung adenocarcinoma transcript-1
MCC	Merkel cell carcinoma
MCMV	Mouse cytomegalovirus
MCV	Merkel cell polyomavirus
MHC	Major histocompatibility complex
MMTV -LTR	Mouse mammary tumor virus long terminal repeat
mRNA	Messenger RNA
MSG	Minor salivary glands
MT Ag	Middle T antigen
NCCR	Noncoding control region
OC	Oral candidiasis
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer solution
PCR	Polymeraase chain reaction
pi	Post infection

PIV	Parainfluenza virus
PP2A	Structural subunits of protein phosphatase
pSS	Primary Sjogren's syndrome
PyV -MT	Polyomavirus middle T
RKV	Rabbit kidney vacuolating virus
RNA	Ribonucleic acid
RPV	Rat polyomavirus
RT-PCR	reverse transcriptase PCR
SGD	Salivary Gland Diseases
SLE	Systemic Lupus Erythematosus
SS	Sjogren's syndrome
SSH	Suppression subtractive hybridization
sSS	Secondary Sjogren's syndrome
ST Ag	Small T antigen
STMV	Stump- tailed macaque virus
TAR	Transactivation response
TLR	Toll- like receptor
tRNA	Transfer RNA
vDNA	viral DNA
VP	Viral protein

CHAPTER 1

INTRODUCTION

Salivary Gland Diseases

Salivary gland disorders cause significant morbidity in both immune competent and immune suppressed populations. While some of these diseases have been well characterized, the etiology of others remains an enigma. Salivary gland diseases are generally characterized by pain or swelling of saliva-producing tissues in the mouth. Saliva-producing tissues include three major salivary glands, the parotid, sublingual and submandibular glands. Saliva from the major salivary glands enter the mouth through ducts at various locations in the mouth. Additionally there are numerous minor salivary glands distributed throughout the oral cavity.

Two major manifestations of salivary gland disorders include reduced saliva production and salivary gland swelling. Dry mouth or xerostomia occurs when saliva production is insufficient or almost nonexistent. Xerostomia predisposes the individual to oral candidiasis, rampant tooth decay, and progressive periodontal disease [1]. Salivary gland swelling can be caused by blockage of one of the ducts that transport saliva. A sialolith or stone is the most common cause of blockage, however infectious diseases such as hepatitis and AIDS and autoimmune diseases including Sjögren's syndrome, diabetes mellitus, and sarcoidosis may be accompanied by swelling of the major salivary glands. Salivary gland enlargement can also result from cancerous or noncancerous tumors in the salivary glands.

HIV-associated Salivary Gland Disease (HIV-SGD)

HIV-SGD is AIDS defining in pediatric HIV infection and is increasing in the adult HIV population. The term HIV-SGD was first coined by Schiodt et al [2] to describe an

enlargement of major salivary glands and/or by complaint of xerostomia in the absence of xerogenic agents, medications, and diseases known to cause xerostomia in HIV patients [3]. In HIV-SGD, quantitative changes occur in the saliva, such as lower secretory rates of sodium, calcium chloride, cystatin, lysozyme and total anti-oxidant capacity which alters the homeostasis of the oral cavity and results in significant morbidity during the progression of HIV disease [4]. Typically, HIV-SGD presents as a unilateral or bilateral diffuse soft swelling resulting in facial disfigurement, and may be associated with pain. Histologically, HIV-SGD is characterized by hyperplastic intraparotid lymphnodes and/or lymphatic infiltrates within the salivary gland tissue. This lymphocytic infiltrate consists predominantly of CD8 T cells which are also CD29+, indicative of a memory cell phenotype. Recent histochemical analysis has revealed the location of the infiltrate as periductal with acinar atrophy, ductal dilation and mild to moderate fibrosis with collagen deposition [5-6]. The minor salivary glands are also affected in HIV-SGD with labial salivary glands demonstrating features of sialadenitis.

HIV-SGD has been universally established as among the most important AIDS-associated oral lesions. Oral lesions are important clinical indicators of HIV/AIDS by suggesting HIV infection in the undiagnosed individual, indicating clinical disease progression and predicting development of AIDS [7]. CD4 cell counts and high HIV viral load have been strongly associated with specific common oral lesions suggesting that oral disease manifestation may serve as a potential clinical marker for HIV viremia and disease progression. Since the introduction of highly active antiretroviral therapy (HAART) in the mid-1990s, the prevalence of types of oral cavity infections of HIV-infected patients has changed across time, with hairy leukoplakia (HLP), oral candidiasis

(OC) and oral Kaposi's sarcoma (KS) decreasing and the incidence of HIV-SGD increasing [8-9]. For example, in an HIV-infected North Carolina population, HIV-SGD has gone from 1.8% in an early cohort to 5.0% in a late cohort [9]. This increase in HIV-SGD after the introduction of HAART drug regimen is reminiscent of previously described cases of immune reconstitution disease (IRD). In HIV infected patients, IRD is an adverse effect of a restored immune response during initial treatment with HAART [10]. Rizos et al described a patient on HAART with excellent immunological and virological response and parotid gland enlargement that may have reflected immune reconstitution [11]. IRD typically occurs as a result of a pre-existing opportunistic infection or a subclinical infection becoming "unmasked" upon the introduction of HAART treatment when HIV viral load decreases and CD4 count increases. Therefore, HIV-SGD presenting as an IRD implies that an infectious agent may play a role in its etiology.

In a prospective study by Mastroianni [12], the association of another salivary gland disease, Sjogren's Syndrome (SS) with the progression of HIV-related disease in a cohort of HIV patients treated with HAART was evaluated. In this study, out of the 150 HIV-positive patients treated with HAART, four were positive for SS whereas none of the HIV positive patients not treated with HAART fulfilled the diagnostic criteria for SS. In conclusion, the study suggested that HAART treatment may play an important role in the aetiology of SS, and that SS may be a new and important complication of long-term HAART regimens[12]. Interestingly, the majority ($\frac{3}{4}$) of these patients did not express the autoantibodies typical of SS and thus probably had HIV-SGD. This study highlights the clinically identical nature of the two diseases and provides further evidence that HIV-

SGD increases with HAART therapy. As more people receive HAART therapy the potential for increasing incidence of HIV-SGD grows.

In developing countries the incidence of HIV-SGD has been reported to be as high as 48% of HIV-1 infected patients [13]. Parotid gland lesions are generally much larger and more disfiguring in pediatric patients than in adults, and in most children xerostomia is not usually associated with the parotid swelling, although in adult patients it is a fairly common complaint. In both adults and children the lesion often occurs in conjunction with generalized lymphadenopathy. Reasons for the differences between the adult and pediatric manifestations are not yet understood [14]. In children with HIV infection it has been shown that the presence of salivary gland enlargement is associated with less rapid progression to death than is found in those with OC or herpes simplex. The time to death averages 5.4 years, compared with 3.4 years with OC [15]. This may be due to a protective effect of the CD8 T cells that predominate in the lymphocytic infiltrate in the salivary glands in that condition and are part of diffuse infiltrative lymphocytic syndrome (DILS) [8, 16-17]. The HIV-SGD disease process is of particular interest because in 1-2% of patients, malignant lymphomas have been described in association with these glandular lesions making this disease a premalignant lesion [18-19].

Diffuse infiltrative lymphocytic syndrome (DILS)

HIV-SGD reflects a localized manifestation of DILS that occurs in the parotid gland of HIV- positive persons. DILS was first described by Itescu et al [20] and is characterized by salivary and lacrimal glandular swelling and sicca symptoms of varying intensity accompanied by persistent circulating and visceral CD8+ lymphocytic

infiltration of parotid and lacrimal glands, lungs, kidneys, nerves, muscles, and lymph nodes, in association with HIV infection. DILS has also been designated as a cystic lymphoid hyperplasia of AIDS and is a close phenotypic mimic of Sjogren's Syndrome in terms of sicca symptoms, salivary glandular enlargement, histology and predisposition to develop non-Hodgkin's lymphoma. The difference between DILS and Sjogren's syndrome, however lies in the frequent occurrence of extraglandular sites of lymphocytic infiltration in DILS, the nature of the infiltrating lymphocytes, that is, CD8 in DILS versus CD4 in Sjogren's syndrome, and the scarcity of serum autoantibodies in DILS [17]. Several conditions that look similar to DILS and can be misdiagnosed include salivary gland tumors, parotitis, tuberculosis, sarcoidosis and sialadenosis. [21]. Similar to HIV-SGD, patients with DILS appear to be at a significantly increased risk of malignant lymphomas [22].

Sjogren's Syndrome

Both HIV-SGD and DILS present Sjogren's syndrome-like conditions in the oral cavity. Table 1.1 shows a comparison of salivary gland disorders: Sjogren's syndrome and HIV-SGD. Sjogren's syndrome (SS) is an autoimmune inflammatory disorder characterized by an infiltration of CD4 lymphocytes in the exocrine glands, especially the lacrimal and salivary glands with extraglandular manifestations in approximately 30 percent of cases. SS prevalence in the general population is largely unknown however women are affected nine times more frequently than men. Although SS was first described in the late 1800's its etiology is currently unknown. Genetic, infectious, endocrine and psychoneuroimmunological factors have however been postulated to play

a role in disease manifestation. SS is typically diagnosed either as primary SS (pSS) or secondary SS (sSS). Both classifications of SS manifest as a systemic disease with several organs affected including, kidneys, lung, blood vessels, skin and muscles and 5-10% of patients develop malignant lymphoproliferative disease. However, in sSS in addition to the above described manifestations various autoimmune diseases such as rheumatoid arthritis, systemic sclerosis, systemic lupus erythmatosus and myositis also occur. SS patients typically are also positive to Ro and La autoantibodies. Patients with autoantibodies have an increased risk to develop parotid swelling, respiratory symptoms, lymphadenopathy and lymphoma. Whereas anutoantibody negative patients though unlikely to develop significant systemic features are still at risk of developing connective tissue diseases distinct from SS. [12].

Table 1.1. Comparison of salivary gland disorders:

HIV-associated Salivary Gland Disease vs. Sjogren's Syndrome

HIV-SGD/DILS	SS
<ul style="list-style-type: none"> • an AIDS defining illness in pediatric HIV infection, affecting up to 60% of children worldwide • incidence is increasing in the adult HIV population and post HAART has become a hallmark of HIV-associated immune reconstitution syndrome • Infiltration of CD4+ T cells 	<ul style="list-style-type: none"> • the second most common autoimmune rheumatic disease, affecting ~1 – 2 million Americans, affects women 9x more frequently than men • EBV, KSHV, Coxsackie and HTLV have been investigated as potential etiologic agents • Infiltration of CD8+ T cells

SGD's: infectious etiologic agents?

In HIV-SGD, epidemiologic data point to an antigen driven process. Basu et al [23] described a prospective study in which he observed that the epidemiology, clinical presentation and certain extraglandular manifestations of HIV-SGD/DILS have changed, concomitant with the introduction of HAART, suggesting that DILS is an antigen (viral)-driven response and the primary treatment for it is anti-HIV therapy[23]. Further evidence alluding to an infectious etiologic agent is the data showing different rates of HIV-SGD in children (20-47%) versus adults (3-7.8%) [19]. In Chapter 2 of this thesis, we postulate that this may indicate primary viral infection in children versus residual immunity in adults. Further, the infiltrating lymphoid cells present during HIV-SGD point to an antigen driven MHC-determined host immune response [17]. That the lymphocytosis within HIV-SGD is phenotypically an antigenically driven response may also implicate an infectious agent present during HIV immunosuppression.

It has been postulated that SS autoimmune disease is primed by viral infection. There is evidence that the type 1 interferon pathway is highly stimulated locally and systemically in SS [24]. Also it has been suggested that viruses may initially infect the gland leading to production of type 1 interferon by plasma dendritic cells. Interferon would then activate the adaptive immune system. Activation of the adaptive immune response would then result in auto-antibody immune response even in the absence of virus. In the following section we explore previously published associations of viral infection and SS.

Viruses and salivary gland disorders

There is a long association between viral infections and diseases of the salivary gland. In general, viral infections of the salivary gland may result in reduced saliva production and/or swelling. Mumps is the most common salivary gland infection caused by paramyxovirus. Paramyxovirus is transmitted by droplet infections carried in the saliva. There is a primary viral infection of the oral cavity and a multiplication of the virus in the upper respiratory tract and the regional lymphnodes. Paramyxoviral infection causes bilateral swelling of the parotids, although the other salivary glands may also be affected. However, subsequent to vaccine development the incidence of mumps has drastically been reduced.

Several viruses, including DNA, RNA and retroviruses have been considered important co-factors in the development of SS [12]. The two DNA viruses that have been studied in association with pSS are cytomegalovirus (CMV) and Epstein–Barr virus (EBV)[25-31]. Overall, the data regarding CMV and EBV as causative agents for SS are contradictory and, because pSS does not occur in most cases of viral reactivation in vivo, the link between reactivation and autoimmunity induction remains to be established. Two RNA viruses have been detected within the salivary gland of SS patients: Hepatitis C and coxsakievirus, both of which require more investigation before they are classified as being the etiologic agent of SS [32-33]. Interestingly, four different retroviruses have been associated with pSS: human T lymphotropic virus type 1 (HTLV-1), human immunodeficiency virus (HIV), intracisternal A type particles and human retrovirus 5 [34-37]. However, only HTLV-1 and HIV produce a Sjogren's-like syndrome with significant pathologic and clinical differences from pSS.

In HIV positive patients, Rivera et al. identified HIV p24 protein expressed in the salivary glands by immunohistochemistry technique [6]. Rivera's group was also able to identify Epstein-Barr virus (EBV) protein but not cytomegalovirus (CMV) by immunohistochemistry in the salivary glands of HIV patients [6]. Human herpesvirus 6 (HHV-6) and HHV-7 were also demonstrated in salivary glands and in saliva in HIV positive persons [38] however their presence has not been attributed to etiological importance for salivary gland lesions. The lymphocytic infiltration observed in HIV-SGD/DILS may be an antigen driven event due to the presence of infection of the salivary gland. Other studies have also looked at the role of CMV, EBV and Kaposi's sarcoma herpes virus (KSHV) in Benign Lymphoepithelial Cysts (BLC) of the parotid gland. Yen et. al however, concluded that CMV and KSHV were not associated with BLCs, although EBV was found more frequently in BLC than in normal parotid controls [39]. Table 1.2 lists viruses that have been detected within the salivary gland and their disease association.

Multiple viruses are also associated with SGD development in animals. The ability of sialotropic murine CMV (MCMV) to induce acute and chronic salivary glandular disease in an autoimmune-prone mouse strain was recently described [40]. Encephalomyocarditis (EMC) virus-induces sialodacryoadenitis in mice [41]. Papovaviruses have also long been associated with salivary gland disease development in experimental animals. Murine infection with polyomaviruses is associated with parotid gland tumorigenesis and has been implicated in human carcinogenesis [42]. Athymic nude mice were found to develop a wasting disease accompanied by parotid sialoadenitis with intranuclear inclusion bodies in acinar and ductal epithelial cells. Intranuclear inclusions and

cytoplasm of the parotid epithelium were found to express SV40 antigens [43]. Salivary epitheliomas induced by injection of neonatal mice with mouse polyoma virus were infiltrated with immature T lymphocytes[44]. This infiltration is reminiscent of SGD. These infiltrates were also described in tumors that arose at the site of inoculation in newborn guinea pigs inoculated with SE polyomavirus [45]. A transgenic mouse model in which the Polyomavirus middle T (PyV-mT) oncogene is expressed under the control of the mouse mammary tumor virus long terminal repeat (MMTV-LTR) has been described which displays clinically observed salivary gland enlargement similar to HIV-SGD [46]. PyV-mT produces a tumorigenic phenotype when expressed alone in cell lines and animals making it a very useful tool for studying oncogenic signaling pathways [47].

As suggested by the body of work reviewed above viral infection is tightly associated with salivary gland pathology (Table 1.2). With the exception of mumps virus and PIV, the majority of these human studies only detect viral nucleic acid but do not determine whether salivary gland tissues are actually permissive for these agents. The potential for a viral etiologic agent in HIV-SGD and potentially SS are highly feasible. This possibility is explored in Chapters 2 and 3 of this dissertation.

Table 1.2 Viruses detected in the salivary gland

Virus	Associated disease(s)
Paramyxovirus	Mumps[48]
Human Immunodeficiency virus (HIV)	HIV-SGD[49], SS[35]
Epstein Barr virus (EBV)	BLC[39], Salivary gland tumors[50], SS[26]
Cytomegalovirus (CMV)	Sialdentitis[51]
Coxsakiavirus	SS [33]
Human T-lymphotropic virus (HTLV)	SS[35, 37, 52]
Human herpes virus 6 (HHV-6)	Parotitis [38, 53]}
Human herpes virus 7 (HHV-7)	Parotitis[38, 54]
Kaposi sarcoma virus (KSHV)	Warthin's tumor[55] SS associated parotid MALT lymphomas[56]
Influenza	Parotitis[57]
Parainfluenza virus (PIV)	Parotitis[58]
Guinea Pig CMV	Guinea pig CMV infection[59],
Hepatitis C	SS reviewed in[32]
Mouse CMV (MCMV)	Acute and chronic SGD[40]
Mouse polyomavirus (PyV)	Mouse parotid tumor[60]
Encephalomyocarditis (EMC)	Sialodacryoadenitis

Differentially expressed genes in salivary gland disorders

An understanding of the gene expression profile specific to salivary gland disorders is likely to provide a framework for a more selective investigation of the molecular mechanisms of the disease, to identify an etiologic agent and for the identification of therapeutic targets. Several studies investigating differential gene expression in salivary gland disorders, specifically SS, have been performed including the use of mouse models and human biopsies. In studies performed by Hjelmervik et. al [61] and Gottenberg et. al [62] using microarray analysis, they observed over-expression of many immune response genes in SS, including interferon inducible genes for example interferon-stimulated transcription factor 3 γ (ISGF3G) and IFN-induced transmembrane proteins (IFITM1). These genes were induced by either IFN- α or IFN- γ in vitro in salivary gland epithelial cell cultures. The latter group also found significantly increased expression of toll-like receptor (TLR) 9 and TLR8 in salivary gland biopsies from SS patients [62]. TLR8 and TLR9 sense nucleic acids (single-stranded RNA and prokaryotic unmethylated CpG-DNA, respectively) on endosomal membranes. The identity of the interferon-inducing nucleic acids in the SS salivary glands remains elusive but suggests the potential for the presence of an infectious agent.

Unpublished data from our lab have also identified deregulation of several genes in biopsied salivary glands from HIV-SGD patients compared to healthy controls using micro array analysis and subtractive suppressive hybridization (SSH) techniques. Some of the genes modulated were consistent with the activation of type 1 interferon response elements including MHCII, interferon inducible genes including cytochrome P450 and IFITM1 which are consistent with an antigen driven immune response.

In addition, 25% of the modulated genes detected by SSH had unknown function. The unknown gene with the most number of clones detected was a novel non-coding RNA called metastasis-associated lung adenocarcinoma transcript 1, MALAT-1. The MALAT-1 gene, also known as α gene, is found on chromosome 11q13 and is well conserved among the mammalian species [63]. The MALAT-1 transcript has been shown to localize to the nucleus [64] and currently has no known function except that its 3' end can be processed to yield a tRNA-like cytoplasmic RNA[65]. MALAT-1 is widely expressed in normal tissue and while little is known about its function, appears to be a potentially generic marker for epithelial carcinomas [63, 66-70]. MALAT-1 has been found to be up-regulated in lung adenocarcinoma metastasis [63], endometrial stromal sarcoma of the uterus [68] and non-hepatic human carcinomas [67]. MALAT-1 up-regulation has also been shown to be a predictor of unfavorable osteosarcoma drug therapy outcomes [66]. Chapter 4 of this dissertation investigates the potential function of MALAT-1 in salivary gland disorders.

Polyomaviruses

As suggested by the body of work reviewed above, polyomaviral infection in animal models are associated with salivary gland pathology. Rise in the incidence of Polyoma viruria has been observed in HIV infected individuals[71], therefore it is easy to imagine that polyomavirus, BKV which infects approximately 80% of the population can be reactivated in immune compromised HIV and SS patients and infect the salivary gland resulting in salivary gland pathology.

Eighteen members of the *Polyomaviridae* family have been identified and all have similar size capsids constructed from three viral capsid proteins. All have genomes of about 5,000 base pairs and display a similar genomic organization. Many regions of their genomes are highly conserved, demonstrating that they have descended from a common ancestor. Different family members of the genus infect different species of mammals, including humans, other primates, rodents, rabbits, as well as birds. Most of these viruses however, display a narrow host range and do not productively infect other species. Table 1.3 lists the members of the *Polyomaviridae* family and their natural hosts.

The first human polyomaviruses were identified in 1971 by two independent research groups. Gardner et al isolated BK virus (BKV) from the urine of a renal transplant patient with the initials B.K. [72], while Padgett et al isolated JC virus from the brain of a Hodgkin's lymphoma patient with the initials J.C who was also diagnosed with progressive multifocal leukoencephalopathy [73]. Most people worldwide become infected with and acquire antibodies to these viruses during childhood, with no apparent disease manifestations. These viruses then appear to lie dormant in the cells of a subset of infected people, and reactivate during

Table 1.3 The Polyomaviruses

Virus	Host species
Mouse polyomavirus (MPyV)	Mouse
Simian virus 40 (SV40)	Rhesus monkey
BK virus (BKV)	Human
WU	Human
KI	Human
Merkel cell polyomavirus (MCV)	Human
JC virus (JCV)	Human
K papovavirus (KPV)	Mouse
Hamster papovavirus (HaPV)	Hamster
Lymphotropic papovavirus (LPV)	African green monkey
Simian agent 12 (SA12)	Baboon
Rabbit kidney vacuolating virus (RKV)	Rabbit
Stump-tailed macaque virus (STMV)	Stump-tailed macaque
Budgerigar fledgling disease virus (BFDV)	Bird
Bovine polyomavirus (ByPV)	Bovine
Rat polyomavirus (RPV)	Rat
Kilham virus (KPV)	Mice
Avian polyomavirus (APV)	Bird

immunosuppression such as in AIDS or transplant recipient patients. Recently, three novel human polyomaviruses have been described, KI, WU and Merkel cell polyomavirus (MCV). KI and WU were identified from patients with respiratory tract infections using large-scale high-throughput screens [74-76]. Merkel cell polyomavirus (MCV) was identified using digital transcriptome subtraction in Merkel cell carcinoma (MCC) and is thought to be the likely causative agent of MCC [77]. The Rhesus macaques virus SV40 was accidentally introduced into the human population via contaminated vaccines, however antibodies against SV40 have been recently detected in individuals who never received the vaccine nor had contact with primates [78]. Therefore SV40 may be considered as a human virus [78-79].

Mouse polyomavirus was the first member of the *Polyomaviridae* family to be discovered and was appropriately named because it caused a variety of different types of tumors including salivary gland tumors.[60]. The initial impetus for studies of the *Polyomaviridae* was their oncogenic potential. Some of these viruses are capable of inducing tumor formation in newborn hamsters. Also, infection of cultured cells in which these viruses cannot grow productively often leads to the malignant transformation of these cells. [80]. Transforming properties of the polyomavirus are encoded by its oncogenic antigens, large, middle and small T antigens, each expressing complementary activities. Studies to understand the oncogenic potential of these viruses have provided fundamental insights into cell cycle regulation, oncogenes, and tumor suppressor genes. Some of these viruses are easy to cultivate in tissue culture and to purify, making them suitable model systems for diverse studies in molecular biology.

The association of polyomaviruses, specifically BKV, in the development of salivary gland disease in experimental animals has led us in Chapter 2 and 3 to investigate their potential role in human salivary gland disorders.

BKV

Virion Structure

BKV is a non-enveloped virus with 40.5- 44 nm diameter icosahedral capsid (Figure 1.1A). The capsid contains three virus-encoded proteins, VP1, VP2 and VP3, surrounding a single molecule of viral DNA complexed with cellular histones in the form of chromatin. These proteins are arranged to form a T=7 icosahedral capsid containing 360 molecules of the major capsid protein, VP1, organized into 72 pentamers. A single VP2 or VP3 molecule associates with each pentamer. Only VP1 molecules are exposed on the outside of the virion. VP1 contains a small groove that interacts with gangliosides GM1, GD1b and GT1b, all of which contain an α 2-3-linked sialic acid residue [81-82] on cell surfaces. The virus particle is 88% protein and 12% DNA and has a sedimentation coefficient of 240S in sucrose density gradients. Because they lack envelopes, the virus particles are resistant to lipid solvents. They are also resistant to heat inactivation. Virions have a density of 1.34g/ml in cesium chloride (CsCl) equilibrium density gradients, whereas empty capsids have a density of 1.29 g/ml [80].

Genome Organization

The genome is a closed circular double-stranded molecule of DNA approximately 5.3kb in length (Figure 1.1B). BKV shares a 75% sequence identity with polyomavirus

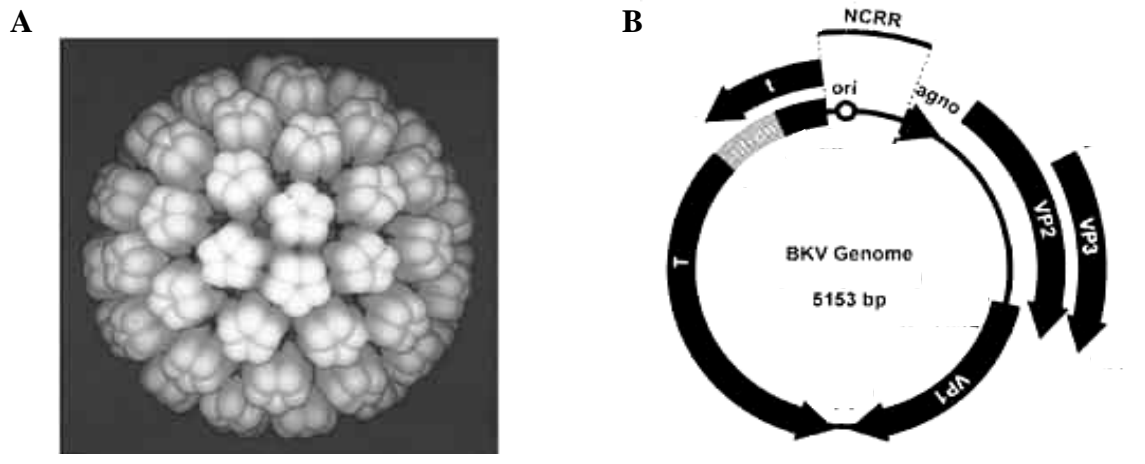
JCV and a 70% identity with SV40 [83-84]. Physical maps of the BKV genome were quickly generated in the 1970's [80] with the advent of restriction endonucleases. BKV's circular genome can be divided into three functional regions: (i) the early region encodes the regulatory proteins large tumor antigen (LTag) and small tumor antigen (STag); (ii) the late region encodes for the structural capsid proteins VP1, VP2, VP3 and the agnoprotein; and (iii) the noncoding control region (NCCR) which harbor the origin of replication and the sequences involved in the transcriptional regulation of both the early and late genes. The early region is by definition, that portion of the genome transcribed and expressed early after the virus enters the cell, and it continues to be expressed at late times after infection, after the onset of viral DNA replication. The mRNAs encoding for LTag and STag are produced by alternative splicing from a common pre-mRNA. Removal of the LTag intron splices the first exon with the next exon allowing translation of LTag. Alternatively, retention of the intron allows translation to reach a termination codon within the intron resulting in STag. LTag is a multifunctional protein with distinct domains fulfilling different roles. Major properties of LTag include its helicase activity and its ability to bind host cell regulatory proteins like the retinoblastoma protein family, p53, pRb and others. These activities are important as they control cell function and undermine the infected cell's destiny for apoptosis. Thus LTag controls both viral DNA replication, early and late gene expression, and interferes with host cell transcription factors. STag promotes cell cycle progression by associating with the catalytic (C) and structural subunits (S) of protein phosphatase 2A (PP2A) [85-86]. This interaction displaces the B subunit of PP2A resulting in inhibition of phosphatase activity [85-86]. Inactivation of PP2A in turn results in the activation of the mitogen-activation protein kinase [87] and

stress-activate protein kinase [88] pathways as well as pathways that use nuclear factor- κ B (NF- κ B), protein kinase C and phosphatidylinositol-3 kinase (PI-3 kinase)[88] . Most of the studies on STag have been performed with SV40 STag and mouse polyomavirus ST Ag, and it is assumed that BKV STag functions similarly.

The late region of the genome is expressed efficiently only after viral DNA replication begins, although low levels of transcription of the late region occur early after infection as well. The late regions of these viral genomes encode the three capsid proteins, VP1, VP2 and VP2 (Figure 1.1B). As with early mRNA, late mRNAs are generated from a common pre-mRNA by alternative splicing. The coding regions of VP2 and VP3 overlap, with VP3 sequences being a subset of VP2 sequences. The coding region of the N-terminus of VP1 overlaps that for the C-termini of VP2 and VP3, with translation of VP1 being from a different reading frame than VP2 and VP3. A fourth late protein called agnoprotein is also encoded by BKV. This 62-71 amino acid protein is encoded by the leader region of the late viral mRNA and accumulates in the perinuclear region during the late phase of the infection cycle. It was originally named agnoprotein because its function was unknown. It appears however, to facilitate the localization of the major capsid protein to the nucleus and may also enhance the efficiency with which virus spreads from cell to cell [80].

The promoters and enhancers for transcription are located close to the origin of replication. Together, the promoters, enhancers and origin are referred to as the NCCR. Transcription extends bi-directionally from initiation sites near the origin, with early and late messenger RNAs (mRNAs) being transcribed from opposite strands of the viral

genome. Thus the early region extends from the origin to a site about halfway around the genome.



(Adapted from Cubitt 2006 {Cubitt, 2006 #280},
http://www.brown.edu/Research/Atwood_Lab)

Figure 1.1 Virion structure and genomic organization of BK virus.

A. Representation of the structure of the BKV virion. Note that the shell is made up of 72 pentamers of VP1. Twelve of these lie on icosahedral fivefold axes and are surrounded by five other pentamers. The remaining 60 pentamers are surrounded by six other pentamers

B. Genomic map of BK virus Dunlop strain. The origin of replication (ori) and transcriptional regulatory region (NCRR) is at the top. The early region extends counterclockwise and the late region clockwise from the top. The regions encoding viral proteins are in black and the position of the intron in grey.

Life Cycle

Interaction of a virus with its cell surface receptor is the initial step during infection of a host cell. In the case of BKV, the viral capsid VP1 interacts directly with $\alpha(2,3)$ -linked sialic acid to bind to and invade permissive host cells [81, 89]. The BKV viral receptor, $\alpha(2,3)$ -linked sialic acid, is believed to interact with the binding cleft between the BC1 and BC2 loops of the major capsid protein of BKV VP1. This speculation is based on the high structural similarity between VP1 of BKV and the crystallized VP1 proteins of mPy and SV40, which both bind to their receptors at their exposed BC loops [90-94].

Like SV40, BKV uses caveolae-mediated endocytosis to be delivered to the ER for uncoating. [95]. An acidification step is required once the virus is inside the caveolae. Lysosomotropic reagents that neutralize the pH of intracellular vesicles have been shown to prevent production of BKV viral proteins [95]. BKV's entry into the nucleus has not been well described although it is believed to traffic through the Golgi to the ER and then accumulate at perinuclear regions [95-96].

Traditionally, basic research on BKV has been conducted using the African green monkey kidney cell line (Vero) which demonstrated a role for sialic acids, galactose, and polysialylated gangliosides as a receptor for BKV infection of Vero cells and in virus binding to red blood cells [97-99], as well as studies on viral trafficking. Most recently however a human proximal tubule epithelial (HPTE) cell culture system was developed to investigate BKV pathogenesis in human cells [100]. BKV's receptor on these HPTE cells indicate a prominent role for the gangliosides GD1b and GT1b [81].

The time course of infection by polyomaviruses primarily depends on two parameters: multiplicity of infection and growth state of the cell. Infection proceeds more rapidly at higher multiplicities of infection than at lower multiplicities. This most likely reflects the fact that critical levels of the viral early proteins are attained earlier when more viral genomes are available for transcription to produce the viral early mRNAs. The other critical parameter is the growth state of the host cell. At the same multiplicity of infection, viral DNA replication begins sooner, and the production of progeny virions is completed more rapidly when cells are growing exponentially than when cells are confluent and in a G₀ state at the time of infection.

The genome and life cycle of BKV has mostly been inferred from studies performed on SV40 and mouse polyomavirus. Therefore it is important that studies on BKV be carried out to determine more clearly how its individual proteins function in its life cycle as well as its role in disease.

Route of transmission and seroconversion

BKV is known to be widespread in the human population, with more than 80% of the world population asymptotically infected [101]. Most people are infected during childhood and up to 90 % of children between ages 5 and 9 are seropositive. As people age, their antibody titers decrease [102-104]. The high incidence of BKV infection raises obvious questions about the mode of transmission from one individual to another. Given the known latency of the virus in the kidney, urine would appear to be a natural vehicle for spread within and between families via the urino-oral route. However specific studies on the early acquisition of BKV infection have not been done. BKV DNA has been

detected in nasopharyngeal aspirates, feces, blood, semen, genital tissues, tonsils, peripheral blood mononuclear cells (PBMC), and normal skin biopsies, hence it is possible that the virus may be transmitted by intimate contact with infected individuals. The possibility of feco-oral transmission has been recently raised by the demonstration of viral DNA in urban sewage[105]. Transplacental transmission of BKV from mother to fetus has also been evaluated however the data is conflicting and controversial [106-109]. Respiratory transmission cannot be ruled out since there is evidence of children with respiratory diseases showing high percentage of tonsillar tissues containing nonintegrated forms of BKV DNA [110]. In conclusion, transmission of BKV is currently not well defined. Data shown in chapter 5 this dissertation suggests that BKV transmission may occur via the oral route.

Pathogenesis, dissemination and persistence

BKV infection typically occurs during childhood, with or without mild respiratory symptoms, followed by a state of non-replicative infection in various tissues, with the urogenital tract as the principal site [111] 112]. BKV DNA has been detected in 30 to 50% of healthy kidneys, primarily in within the cortex and medulla and to a lesser extent in the ureters [113-114].

BKV DNA has also been found in several other cell types including cervix, vulva, prostate, semen and brain tissues [114-119], as well as in peripheral blood mononuclear cells (PBMC) and spleen [101, 117, 120]. It is possible that PBMCs may play a role in persistence and dissemination of BKV in the human host. While BK DNA sequences have been widely detected in many cells types, its replication and pathogenesis have only

been studied within the kidney cell. Chapter 5 of this dissertation explores the potential for BK permissive infection in the salivary gland cell.

BKV-associated diseases

It is not known whether BKV enters a latent state or maintains a low level of viral gene expression and replication within detected cell types and tissues, although intermittent replication must occur as evidenced by periodic excretion of virus in the urine.

Approximately 5% of periodic immunocompetent individuals have BKV viruria as well as approximately 3% of pregnant women have asymptomatic viruria during the second and third trimesters [109, 121-123]. How the host innate, humoral and cellular immune responses control BKV persistent infection remains unknown.

BKV in Immune suppression

BKV-associated nephropathy (BKN)

BKN has been described by Nickeleit et al as ranging from intranuclear viral inclusion bodies to necrosis with denudation of basement membranes within tubular epithelial cells, as a result of BKV replication [113]. Several investigators have studied and reviewed BKN and it has been determined that 1-9% of renal transplant recipients typically develop BKN post transplant [124-126] and of those patients with BKN up to 80% result in graft loss [125, 127].

The mechanism by which BKV reactivates in kidney epithelium of renal transplant recipients is poorly understood. While it has been suggested that immune suppression may play role in viral reactivation this is not always the case as nonrenal

solid organ transplant and other immunosuppressed patients do not develop BKN [112, 128]. Other risk factors including patient age, gender, HLA mismatches, BKV serotype or type of immunosuppressive drug used may play a role in the development of BKN [127, 129-131].

The definitive diagnosis of BKN requires allograft biopsy. One of the difficulties associated with biopsies as a diagnostic measure is sampling error due to the multi-focal characteristic of BKN. Typically a biopsy is accompanied by tests that detect the presence of decoy cells or virus infected cells shed in the urine and viremia [132]. Singh et al has recently described a new method for diagnosing BKN by the detection of "Haufen" (after the German word for "cluster or stack") or clusters of BK virions via electron microscopy in voided urine [133].

Currently there is no approved treatment for BKN. Typically the level of immunosuppression is monitored and polyomavirus-inhibitory drugs such as Cidofovir is administered. Cidofovir is an antiviral nucleoside analog which has been shown to be effective in treating BKN along with reduced immunosuppression [134-136], however at high doses this drug has been shown to be nephrotoxic [137]. Retransplantation is another intervention option for BKN patients although there is the possibility that disease manifestation will not re-occur.

Hemorrhagic cystitis

Another BKV-associated complication is hemorrhagic cystitis (HC) which occurs in more than 10% of bone marrow transplant (BMT) recipients [138-139]. HC diagnosis typically includes dysuria and viruria which occurs at about 2-12 weeks post

transplant[140-141] using both urine cytology and PCR detection of viral DNA [139] . BK virions can be detected in exfoliated epithelial cells and is thought to be caused by viral reactivation in the urothelium [142-143]. The onset and termination of viruria has been shown to correlate with the progression of HC {Ahsan, 2006 #196} [140, 144]. Like BKN, Cidofovir is also being tested as a treatment option for HC patients[145-147].

Human Immunodeficiency Virus

BKV viruria has been shown to increase in HIV positive individuals [148]with some groups showing increased viruria concomitant with a decrease in CD4+ T cell counts[149]. However, increased BKV viremia in HI- positive individuals has not been established [150-151]. In AIDS patients , BKV has been reported to cause fatal tubulointerstitial nephropathy, disseminated pulmonary infection, retinitis and meningoencephalitis[152-155] . Others have reported hemorrhagic cystitis similar to that observed in bone marrow transplant recipients [156-157].

The effect of HIV proteins on BKV replication was investigated by Gorill et. al in vitro. It was demonstrated that HIV proteins Tat and Vpr dramatically stimulated BKV's early promoter, BKV(E) transcription. Using both site-directed mutagenesis analysis and electrophoretic mobility shift assay (EMSA) Gorill et al showed that Tat activated BKV(E) by inducing binding of the NF-kappaB p65 subunit to kappaB motif at the 3' end of BKV(E). In addition, this study identified a sequence within the 5'UTR of BKV(E), BKV(E)-TAR with homology to HIV-1 transactivation response (TAR) element. Using RNA EMSA assays, it was determined that Tat bound to BKV(E)-TAR while deletion of the BKV(E)-TAR sequence prevented Tat transactivation of BKV(E)

transcription. Site-directed mutagenesis analysis of potential Tat-responsive transcriptional motifs complemented by an electrophoretic mobility shift assay (EMSA) showed that Tat activated BKV(E) by inducing binding of the NF-kappaB p65 subunit to a kappaB motif near the 3' end of BKV(E). In addition, a sequence within the 5' UTR of BKV(E) transcripts (BKV(E)-TAR) was identified that is identical to the HIV-1 transactivation response (TAR) element. The BKV(E)-TAR sequence bound TAT in RNA EMSA assays and deletion of the BKV(E)-TAR sequence eliminated Tat transactivation of BKV(E) transcription. This data suggests that HIV Tat can positively regulate BKV(E) transcription by a dual mechanism which has huge implications for and is extremely important in BKV-associated diseases in AIDS patients [158].

Autoimmune disease

BKV has been suggested to play a role in autoimmune diseases such as Systemic Lupus Erythematosus (SLE)[159-160] and Sjogren's Syndrome (unpublished data). SLE is the prototype of systemic rheumatic syndrome and is characterized by production of a wide array of autoantibodies. The majority of these autoantibodies are directed against nuclear constituents or antinuclear antibodies (ANA). In patients with SLE, it has been suggested that BKV infection might initiate autoimmunity by inducing antibodies against DNA and histones [161-163]. Work performed by Christie et al [164] observed that rabbits inoculated with BKV particles produced antibodies directed to both viral structural protein and host histones [164]. In support of these findings, patients with BKV infection with expression of large Tag have also developed anti-DNA antibodies and anti-BKV antibodies which cross react with DNA [162]. As previously described, SS is an

autoimmune disease of which its etiology is unknown. Several viruses have been implicated in playing a role in SS pathogenesis including EBV, CMV, HTLV, Hepatitis C, Picornavirus and most recently coxsackievirus [33, 165]. Studies in our lab have recently detected BKV within salivary glands of SS patients using PCR and immunohistochemistry (unpublished data).

Other polyomaviruses have also been implicated in autoimmune diseases, namely JC virus in multiple sclerosis [166]. Multiple sclerosis is an autoimmune disease in which the body's immune response attacks a person's central nervous system (brain and spinal cord) leading to demyelination. There are conflicting reports of JCV DNA and viremia in patients with MS: several studies report a lack of evidence for JCV infection in MS patients [167-168], while others have found JCV DNA sequences in the cerebral spinal fluid (CSF) of some MS patients but not in healthy controls [169-170].

BKV and Cancer

Viruses of the *Polyomavirinae* family including SV40, BKV, MCV and JCV have been implicated in human carcinogenesis [42]. With advancements in technology, scientists have been able to detect viral DNA in tumor specimens with more precision. These advanced techniques may account for discrepancies observed between past reports of BKV in tumors and present studies. BKV DNA has been detected as integrated into the genome and episomally from many different types of human tumors. Sites include bone, pancreatic islet cells, kidney, urinary tract, prostate and a wide variety of brain tissues [114, 171-177]. Some of the larger studies show data as follows: using Southern blot technique, BKV was detected episomally in 4/9 tumors of pancreatic islet cells and

19/74 brain tumors.[174]. Further work using PCR technique demonstrated BKV DNA in 58/68 of brain tumors, 21/27 osteosarcomas, and 5/13 Ewing's tumors, the same sequences were also found in 13/13 of normal brain tissue, 2/5 normal bone tissue and 25/35 peripheral blood cells [178]. RT-PCR further showed that most of these samples also expressed BKVRNA. Since it is well known that BKV reactivation occurs during immunosuppression, the presence of BKV DNA was also investigated in tumors associated with immunosuppression such as Kaposi's sarcoma (KS). Using Southern hybridization technique BKV DNA was detected in KS at a frequency of 20%.

In most of the studies, the tumors expressed viral RNA and were positive for large Tag by immunohistochemistry. In general, only small amounts of BKV DNA was detected in both human tumors and normal tissues, averaging fewer than one genome per cell. The low copy number of viral genomes suggests that the tumor cells were transformed by a 'hit and run' mechanism, that is, an autocrine-paracrine effect. For example, Tag expressing cells may be secreting growth factors resulting in the recruitment and proliferation of Tag negative cells. [179]. Although much work has been done involving the detection of BKV in human tumors, the causal link between BKV and cancer development has not been established. .

Objectives

Diseases of the salivary glands and the resultant quantitative changes in saliva affect the homeostasis of the oral cavity causing significant morbidity and poor quality of life. Further, HIV associated salivary gland disease has been associated with the development of malignancy. As reviewed in the body of work above, there is little

known with regard to the role of etiologic agents in both HIV-SGD and SS. Based on the epidemiology of HIV-SGD and its behavior we hypothesized that a DNA tumor virus may play a major role in the disease. As polyomaviruses are associated with salivary gland disease in experimental animals, the potential for their involvement in the human disease is significant. Toward better understanding HIV salivary gland pathology and in search of a potential etiologic agent we pursued and achieved the following aims:

1. To determine an etiologic association of DNA tumor virus with salivary gland diseases

i. HIV-SGD

In patients clinically diagnosed with HIV-SGD, BKV genes and genetic products were consistently detected within salivary gland biopsies. In addition, BK viremia and viral shedding into oral fluids were detected in HIV-SGD patients. Thus, we have determined the presence of BKV infection in HIV-SGD and report for the first time that BK polyomavirus is associated with HIV-SGD.

ii. Sjogren's Syndrome

Based on our results of consistently detecting BKV in HIV-SGD, we hypothesized that BKV may also play a role in a similar disease, SS. BKV DNA and protein expression were detected in a subset of SS patients. Further analysis of host cell de-regulation was investigated and a novel transcript MALAT-1, known to be up-regulated by BKV was also shown to be increased in SS, confirming that BKV may be playing a role in SS

disease pathology. Thus, we have determined the presence of BKV infection in SS and report for the first time that BK polyomavirus is associated with SS.

2. To determine how BKV expression modulates host cellular gene expression

This study details the up-regulation of a malignancy-associated transcript (MALAT-1) by BKV in HIV-SGD, in a SGD transgenic mouse model and in vitro and is the first report of a mechanism for MALAT-1 regulation.

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CHAPTER 2

HIV-SALIVARY GLAND DISEASE IS ASOCIATED WITH BK VIRUS

ABSTRACT

HIV associated salivary gland disease (HIV-SGD) causes significant morbidity in those immune-suppressed by HIV. Evidence detailing the epidemiology of HIV-SGD suggests the involvement of a viral opportunist in its pathogenesis yet the specific etiology of HIV-SGD remains unclear. To determine the role for an opportunistic virus as the etiologic agent of HIV-SGD, we hypothesized that HIV-SGD was a manifestation of primary infection or reactivation with a DNA tumor virus during immune suppression. Minor salivary gland tissue from HIV positive patients with HIV-SGD and from HIV-negative individual control subjects was examined. Degenerative PCR, sequence analysis, reverse transcriptase PCR and immunostaining were used for virus identification and characterization. Real-time PCR was used to assay detection of BK shedding and viremia. While herpesviral sequences were not detected, Polyomavirus DNA was consistently detected in HIV-SGD, and sequence analysis confirmed BKV DNA. In 14/14 patients clinically diagnosed with HIV-SGD, BK viremia/shedding into oral fluids and/or BK viral gene products were detected within HIV-SGD tissue while the related Polyomaviruses were consistently negative. This is the first report of BK salivary gland tropism and shedding into oral fluids. We have determined the consistent presence of BKV infection in HIV-SGD and report for the first time that polyomavirus infection is associated with HIV-SGD.

INTRODUCTION

HIV associated Salivary Gland Disease (HIV-SGD) is characterized by enlargement of the parotid gland, complaint of xerostomia in the absence of xerostomic agents or diseases, and by the development of both lymphoepithelial cysts and lymphoma[1]. HIV-SGD is AIDS defining in pediatric HIV infection affecting up to 47% of children worldwide [2]. Like Sjogren's syndrome, HIV-SGD affects both major and minor glands. HIV-SGD-induced lack of saliva production, predisposes affected individuals to rampant tooth decay, oral candidiasis and progressive periodontal disease. Histologically, HIV-SGD is characterized by hyperplastic intraparotid lymph nodes and/or lymphatic infiltrates within the salivary gland [3]. HIV-SGD is also a feature of diffuse infiltrative lymphocytosis syndrome (DILS). DILS in HIV-infected persons is characterized by a persistent circulating CD8+ lymphocytosis. This CD8+ oligoclonal lymphocyte expansion infiltrates multiple organs, including the salivary glands and may reflect a virus driven antigen- response [4].

Thus far the search for the etiology of HIV-SGD has been inconclusive. Some argue that HIV-SGD is caused by autoimmune deregulation pointing to increased epithelial metaplastic cells that may represent an exuberant reaction to HIV [5]. Others argue that immune dysregulation may manifest as autoimmune reactivity resulting in HIV-SGD [6]. Evidence alluding to an opportunist as an etiologic agent is embodied in epidemiologic data showing different rates of HIV-SGD in children (20-47%) versus adults (3-7.8%) [7]. Further, the incidence of salivary gland disease among HIV infected patients increased significantly following the introduction of highly active antiretroviral

therapy (HAART). The disease becomes apparent shortly after induction of HAART, reminiscent of opportunistic infections triggered by immune reconstitution [8].

Interestingly, a similar increase in the incidence of post HAART-linked HPV associated oral warts has been described [9]. Opportunistic DNA viruses are frequent etiologic agents of HIV-associated oral lesions including Epstein-Barr virus (EBV) associated hairy leukoplakia and Kaposi's Sarcoma associated herpes virus linked Kaposi's sarcoma. Viral infections such as EBV and HIV have been implicated in HIV-SGD [10]. Polyomaviruses are also candidate contributory DNA tumor viruses, and include JC, BK and SV40 virus. Polyomaviruses cause parotid gland enlargement in rodents [11]. Mouse polyomavirus was initially named parotid tumor agent, because of the high incidence of salivary gland carcinoma development [12-13]. In humans, the etiological role for these polyomaviruses has not fully been explored.

Based on epidemiologic correlates and the link to lymphoma development, [14] we postulated that HIV-SGD was a manifestation of DNA tumor virus infection. The objective of these studies was to seek candidate infectious etiologic agents. We determined that while herpesviral gene products were not detected in HIV-SGD, polyomavirus nucleic acids/antigens were consistently detected. BKV sequences and gene products were detected using BK specific reagents. Further, shedding was detected and homologous polyomaviral proteins expressed in mouse salivary gland disease displayed subcellular localization similar to HIV-SGD. Collectively, these studies underscore the potential for BKV to be a key etiologic agent in HIV-SGD development.

MATERIAL AND METHOD

Patients, Animals and Sample Collection.

HIV positive patients were recruited from UCSF oral medicine clinic or UNC hospitals Dental clinic or infectious disease clinic to participate in the IRB approved study.

Venopuncture was performed and plasma isolated. Minor salivary gland biopsies were performed, tissues were either paraffin embedded, snap frozen, or embedded in OTC.

Polyoma T transgenic mice with salivary gland enlargement were FVB mice from Charles River Laboratories, Wilmington Mass were generated that express the MMTV long terminal repeat/PyV-mT((Ellies)). Wild type mice on the comparable background were used as controls.

DNA Isolation and Polymerase Chain Reaction .

Tissues were pulverized and ultracentrifuged on a 4M guanidine isothiocyanate-caesium chloride step gradient as previously described (Webster-Cyriaque, 1998). Throat washes were pelleted, cellular content centrifuged and pellets were treated with Qiagen DNAeasy kit. PCR amplifications were performed with 150-200 ng of DNA as previously described (Webster-Cyriaque. 1998) and primers specific for herpesviral terminase, DNA polymerase, polyomavirus T antigen or GAPDH , with amplification proceeding for 38 cycles. (Hargis, AM et al, 1999). Control DNAs were amplified from B958 (EBV), HCMV-infected HEL cells, and BCBL-1 (KSHV) as well as 200 ng of salivary gland DNA samples. (Van Gorder et al, Bergsagel DJ).

Sequencing

Purification was carried out with ExoSap-IT reagent (USB, Cleveland, Ohio, USA) followed by direct sequencing of the large T antigen region using ABI Big Dye® terminator reagents (ABI) and an ABI PRISM 3730 DNA sequencer. Sequences were compared using NCBI Blast2 software \with EMBL entries for the polyomaviruses for BKV (NC_001538), JC virus, Ki, Wu and SV40 and analyzed using Seqscape® software v2.5 (Applied Biosystems). 3-5 separate amplification-sequencing assays were performed per sample. Phylogentic trees were drawn using the neighbor joining method.

RNA isolation, cDNA, Reverse Transcriptase PCR

Paraffin Block RNA Isolation Kit was used for RNA extraction (Ambion) and genomic DNA was removed with DNase I using DNA-free Kit (Ambion), according to the manufacturer's instructions. RNA quality was assessed spectrophotometrically. Reverse transcription was performed with SuperScript™ II (Invitrogen) Reverse Transcriptase Kit according to manufacturer instructions. For both VP1 and Tag primer pairs, a no-template control produced no signals, suggesting that primer-dimer formation and genomic DNA contamination effects were negligible. Amplification results were normalized to B-actin. Real time PCR was also carried out, gene expression values were normalized to the levels of β -actin transcripts, using the $2^{-\Delta\Delta C(T)}$ method, and are presented as the changes (n -fold) in viral gene levels, with the levels in uninfected/mock samples arbitrarily set to 1.

BKV Amplification from Plasma and Saliva.

BKV DNA amplification from plasma/saliva was performed in duplicate using Tag primers and ABI Prism 7000 and SYBER green PCR core reagents kit according to manufacturer's instruction. The threshold of detection was 3-5 copies of the BKV/JC virus plasmids/reaction. 15 copies of the respective plasmid was added to the reaction mixture to rule out inhibition of the PCR in negative results (Figure 3). Investigators performing the PCR analyses were blinded and PCR for viremia was carried out in two different laboratories. Quantitation was achieved by use of standard curve. The mean copy number in the plasma was 10^6 (range 10^2 - 10^7).

Immunohistochemistry

Frozen sections were cut from minor salivary gland (HIV-SGD and healthy control glands) and from mouse parotid glands (transgenic/wild type) for immunofluorescent and confocal analysis. Tissue sections were fixed, blocked, then stained with 1[°] antibodies targeted to Tag or p53 and stained with 2[°] FITC or lissamine rhodamine conjugated antibodies. Slides were Vectashield coverslipped (Vector Laboratories) then subjected to confocal microscopy. Formalin fixed sections were deparaffinized, and washed. Slides were incubated in 3% hydrogen peroxide and blocked and incubated with 1[°] antibodies for Tag/VP1. DAKO LSAB+ peroxidase kit (DAKO Corporation) was used according to manufacturer's specifications.

RESULTS

Polyomavirus DNA detected in HIV-SGD

Bivariate analysis for HIV-SGD development in our population (n=271) revealed that patients on HAART were 4.4 times more likely to develop HIV-SGD and persons with high levels of HIV RNA (> 400) were 3.1 times more likely to develop HIV-SGD (Figure 2.1A). Both lymphocytic infiltration and increased disease incidence while on HAART are typical of immune reconstitution, implicating an opportunist in the genesis of HIV-SGD. Viruses are consistently detected in saliva, particularly Herpesviruses. Degenerative polymerase chain reaction (PCR) was used to detect novel sequences by exploiting highly conserved Herpesvirus and Polyomavirus regions. Highly conserved Herpesvirus terminase and polymerase regions were amplified [15-16]. The herpes terminase/polymerase genes were detected in cell lines infected with EBV, CMV and KSHV. Herpesviral DNA was not detected in 3/3 HIV-SGD patients shown (Figure 2.1B). A viral microarray containing multiple open reading frames from each of the known human Herpesviruses and of high/low risk papillomaviruses failed to detect expression of viral genes in HIV-SGD tissue (Data not shown). Immunohistochemical (IHC) studies of these tissues did not detect EBV and KSHV viral proteins (Data not shown).

A.

	OR	95% CI
HAART	4.4	(1.7, 9.8)
HIV RNA >400	3.1	(1.2, 8.0)

B.

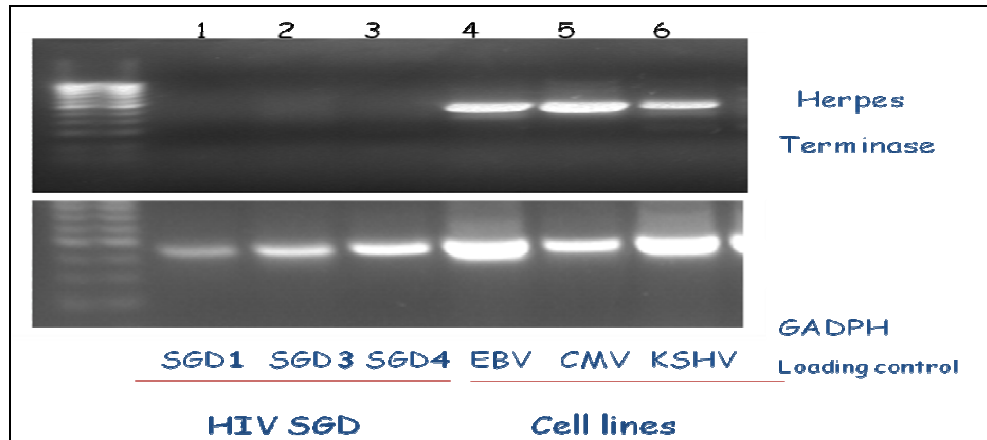
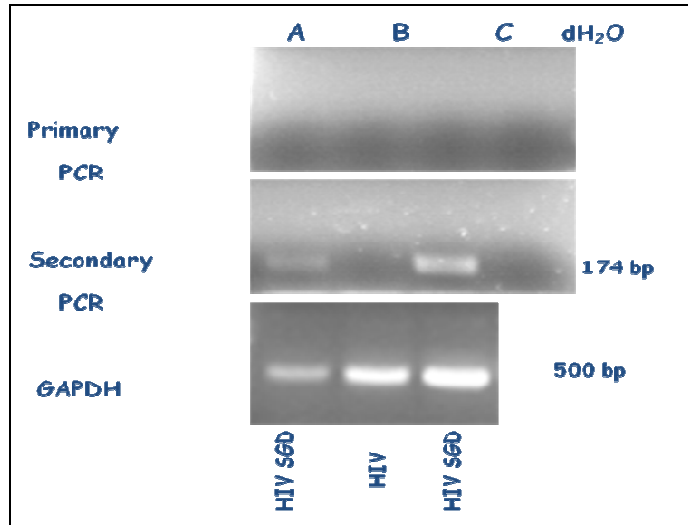


Figure 2.1. Detection of Polyomavirus DNA in HIV-SGD and not Herpesviral DNA.

A) Bivariate analysis for the development of HIV-SGD in our cohort suggests the involvement of an opportunist. B) Control cell lines infected with the herpes viruses: EBV-infected B958 cells, CMV-infected HEL cells and KSHV-infected BCBL1 cells, lanes 4-6 respectively, were readily amplified in this highly conserved terminase region but Herpesviral DNA was not detected in HIV-SGD by degenerate PCR as shown by the lack of bands in lanes 1, 2 and 3.

C.



D.

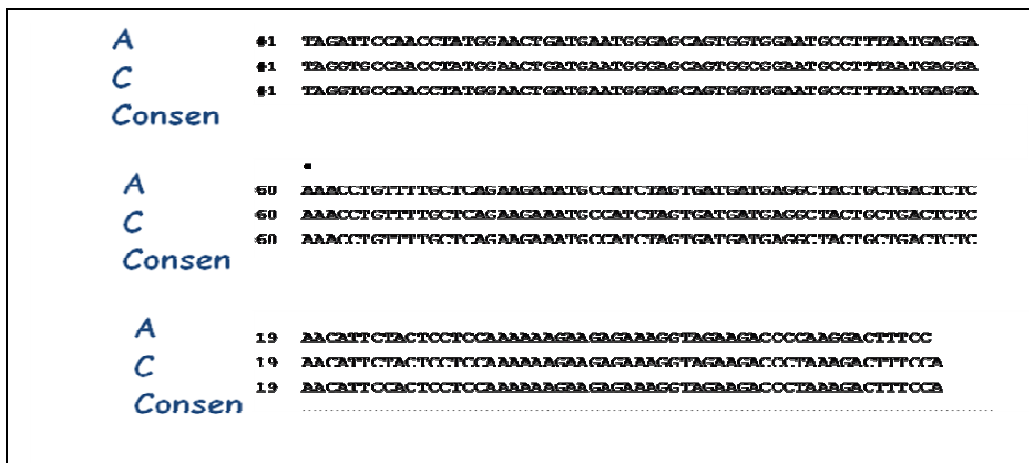


Figure 2.1 (continued). C) Polyomavirus DNA was detected by degenerate PCR in HIV-SGD lanes A and C but not in an HIV patient without the disease, lane B. D) Sequence analysis confirmed the detection of Polyomavirus DNA in HIV-SGD.

Degenerative nested PCR targeted to polyomavirus T antigen (Tag) region detected polyoma DNA in minor salivary glands in 2/2 of the HIV-SGD subjects (Figure 2.1C lanes A and C), but not in a subject who was HIV positive without the disease (Figure 2.1C lane B). Viral gene products of expected size, were cloned and sequenced (Figure 2.1D). Comparisons of amplified regions to known genomes detected 96-99% homology with the Tag regions of SV40, BK and JC viruses. In all, 14 HIV-SGD minor salivary glands and 10 control minor salivary gland tissues were selected for evaluation (Figure 2.2A). A correct size BKV Tag product was consistently obtained in 11/14 samples, while salivary glands from control patients were consistently negative. These 14 HIV-SGD samples were consistently negative for SV40 (Figure 2.2A) and JC virus (data not shown). Multiple pairwise alignment of DNA sequences from the majority of HIV-SGD subjects revealed complete (99%) homology with the BKV MM (shown), Sau 3, PittNP4 and Dunlop strains confirming BKV presence (Coordinates 1206-1377 shown in Figure 2.2B). These sequences were consistently different from SV40, JC and the newly described human polyomaviruses

A.

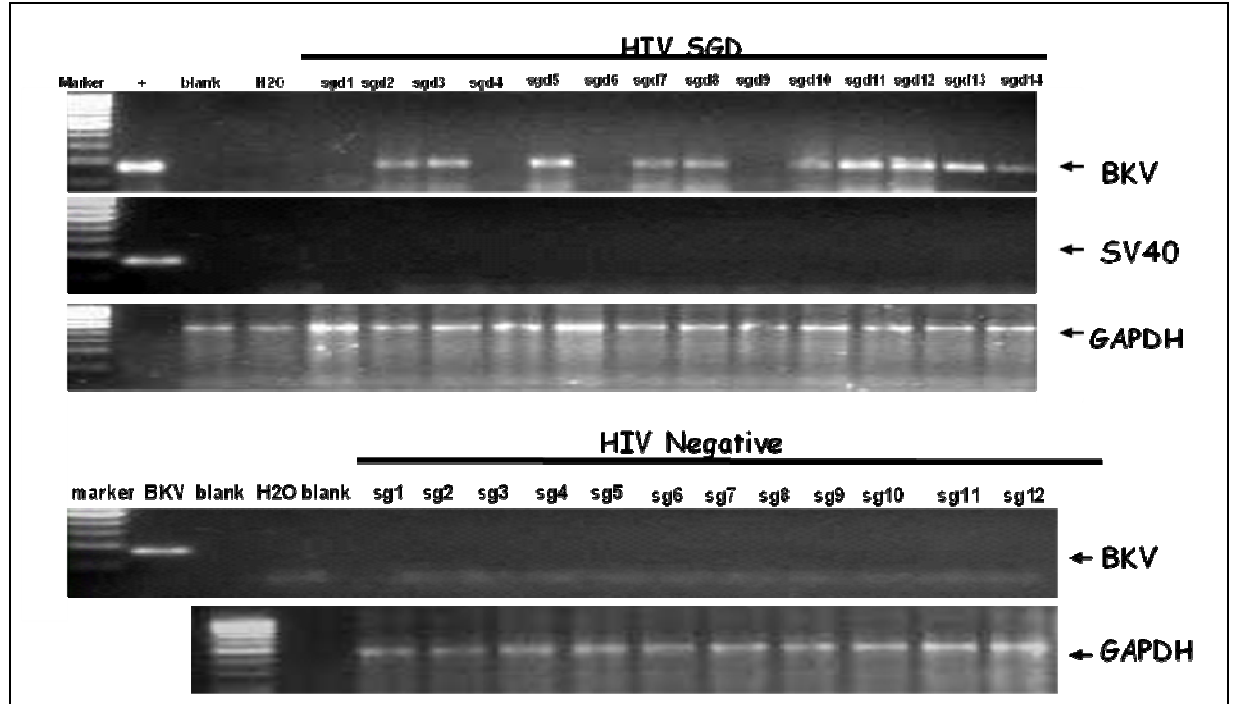


Figure 2.2 Detection of BK virus in HIV-SGD. Amplification from minor salivary glands of HIV-SGD and HIV negative persons with virus specific primers. A) SV40 was not detected in HIV-SGD (SGD 1-14), BKV was consistently detected in HIV-SGD but BKV was not detected in minor salivary glands of HIV negative persons (SG 1-10). (+) positive control H₂O, negative water control.

B.

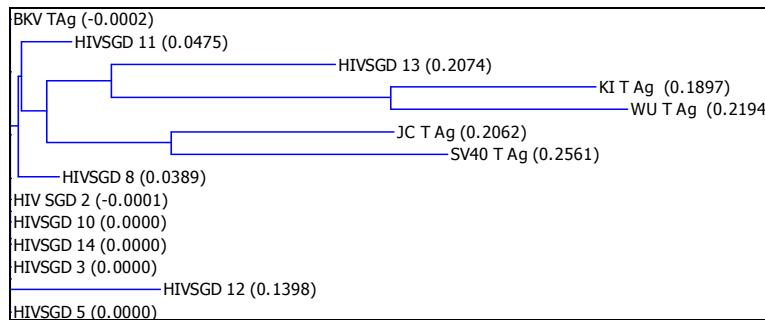
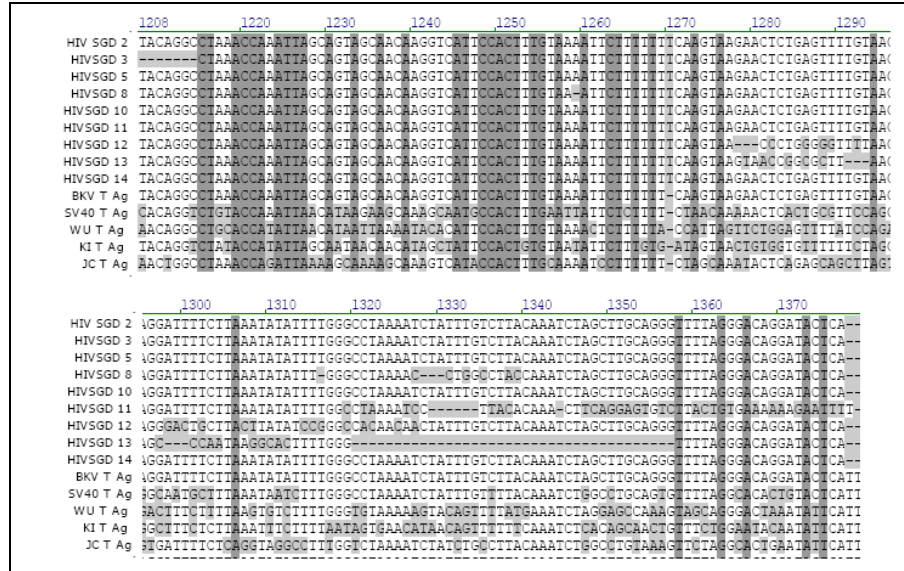


Figure 2.2 (continued). B) Sequence alignment of HIV-SGD PCR products from minor salivary gland tissues matched the highly conserved Tag region of BKV but not SV40, KI, WU or JC. Phylogenetic analysis of sequences from HIV-SGD showed a high degree of relatedness to BKV and not to the other polyomaviruses, WU and KI. Neighbor joining analysis determined the phylogenetic relationship of HIV-SGD sequences to BKV.

BKV gene products detected in HIV-SGD

To determine the state of infection within HIV-SGD, amplification of non-structural and structural BKV transcripts were performed. RT-PCR of RNA from HIV-SGD minor salivary glands detected STag and VP3 in 4 out of 4 diseased patients. Mucous retention cysts (ranula) from HIV negative persons were consistently negative as were the no RT controls (not shown). BKV transfected Vero cells were used a positive control (Figure 2.3A). The histologic expression of BKV proteins was examined in HIV-SGD (Figure 2.3B). The absence of viral protein expression would be strong evidence against the role for BKV in the etiology of the disease. Immunofluoresence and confocal microscopy, detected Tag and its binding partner p53 within HIV-SGD. Immunofluorescence using both T ag monoclonal and T ag polyclonal antibodies determined punctuate perinuclear stain in 4/4 HIV-SGD patients and lack of staining in 2 of the 2 HIV negative individuals (Figure 2.3B polyclonal shown). p53 specific monoclonal and polyclonal antibodies detected distinct intracellular nuclear/perinuclear punctuate staining in 4/4 HIV-SGD patients and not in HIV negative subjects (Figure 4C, monoclonal shown). Confocal microscopy detected p53 and Tag cytoplasmic/perinuclear co-localization within HIV-SGD epithelial cells but not in control glands.

A.

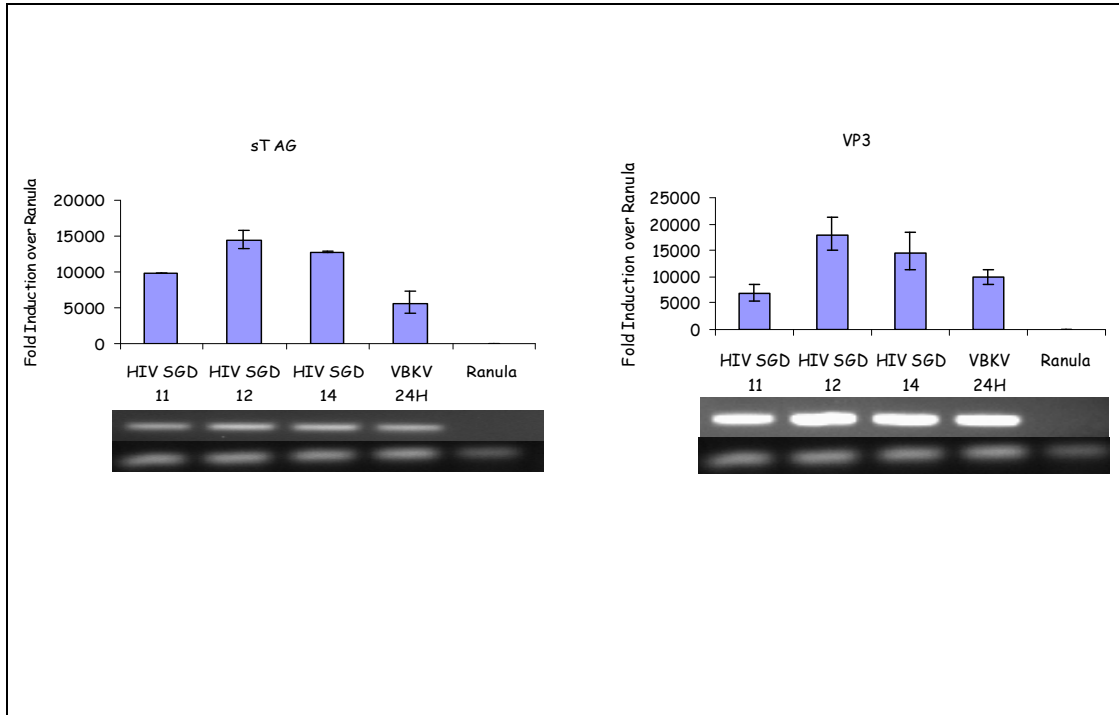


Figure 2.3. Detection of BKV gene expression in HIV-SGD. A) Detection of structural, VP3 and nonstructural, STag BKV transcripts in HIV-SGD and in BKV transfected Vero cells at 24h (VBKV24H) but not in mucous retention cyst, ranula from healthy persons.

B.

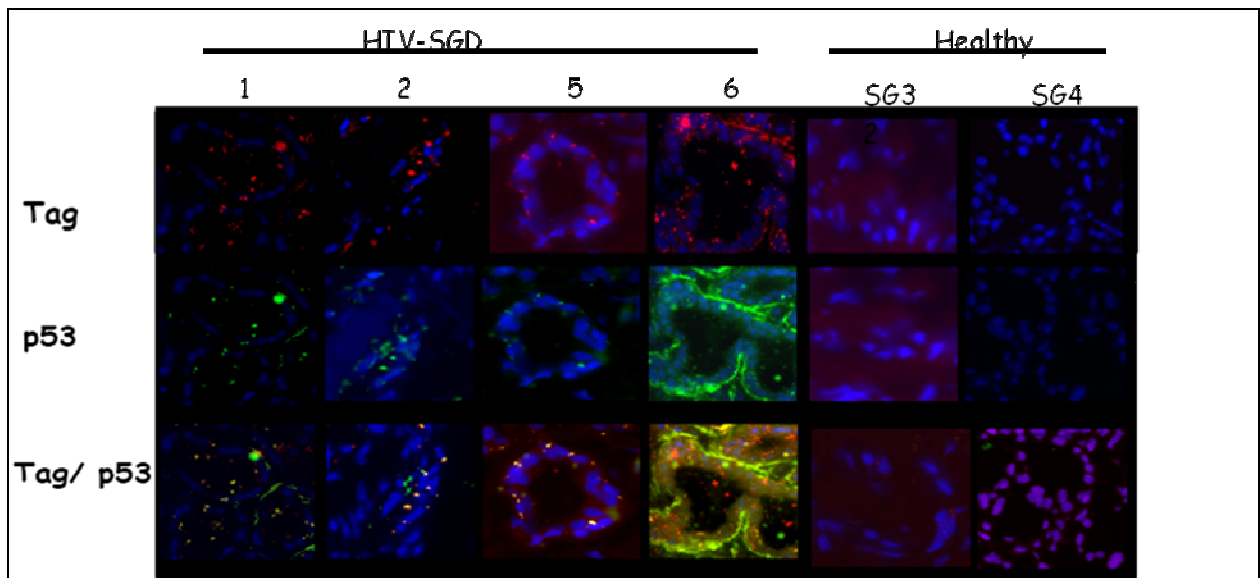
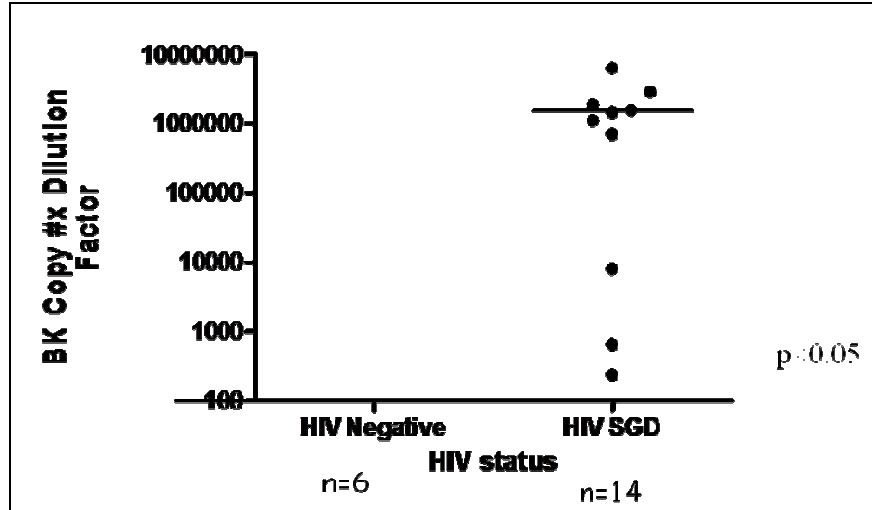


Figure 2.3 (continued). B) Immunofluorescence detects T ag in HIV-SGD subjects 1,2,5 and 6 (polyclonal- red) but not in HIV negative (healthy) subjects SG3 and SG4. p53 (green)(mouse monoclonal-green). Tag and p53 colocalization are also detected in HIV-SGD (yellow).

A.



B.

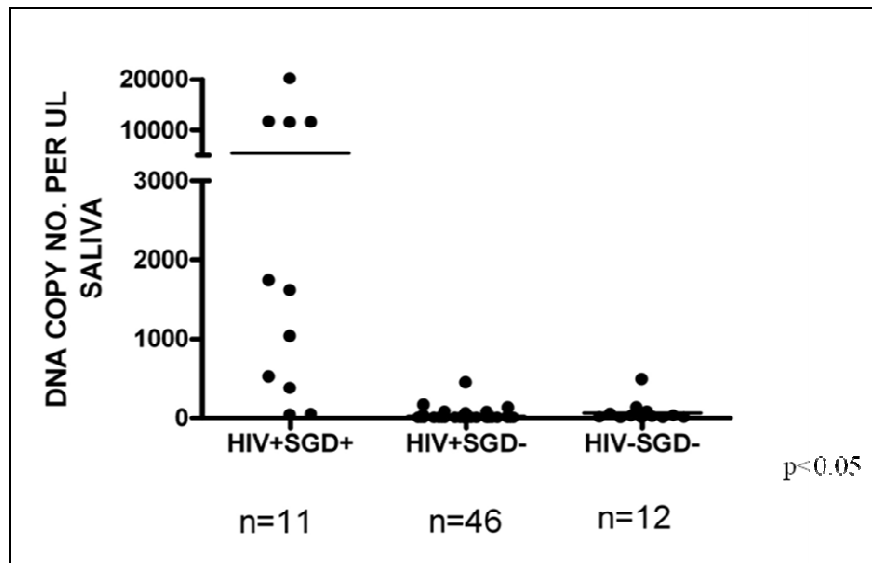


Figure 2.4. Detection of BKV viremia and shedding in oral fluids of HIV-SGD. **A)** qRT-PCR detects elevated BKV levels in the plasma of subjects with HIVSGD (n=14), but not in HIV negative subjects (n=6). **B)** qRT-PCR detects elevated BKV levels in the oral fluids of subjects with HIV-SGD (n=11), but at much lower levels in HIV positive subjects (n=46) without salivary gland disease and in HIV negative subjects (n=12).

BKV DNA detected in blood and oral fluids of HIV-SGD

BKV shedding and viremia were analyzed in HIV-SGD patients. BKV plasma levels, as measured by quantitative real time RT-PCR, were found to be significantly higher in patients harboring the disease. Plasma samples were obtained from HIV-SGD patients within six months of the HIV-SGD diagnosis. In 9 out of 14 patients, the time at which BKV DNA was detected in plasma coincided with the histologic diagnosis of HIV-SGD (Figure 2.3A). Plasma levels in persons with HIV-SGD ranged from 10^2 to 10^6 copies/ml. It is expected that immune suppression itself would increase the opportunity for viremia. Interestingly, in 17 HIV positive, SGD negative patients analyzed in a previous study of our cohort no BKV DNA was detected in plasma [17]. Plasma samples from six HIV negative samples did not exhibit viremia (Figure 2.3A). Salivary shedding was also assessed. While mean BK copy number for HIV-SGD was approximately 5,000 (n=11), those who were HIV positive without the disease (n=46) or were HIV negative had mean BK copy numbers that were significantly lower.

DISCUSSION

Establishment of HIV-SGD etiology may be based on the model offered by Fredericks and Relman of molecular guidelines for establishing causation which states 1) that nucleic acid sequence belonging to a putative pathogen should be present in most cases of infectious disease 2) that fewer/ no nucleic acid sequences should occur in hosts and tissues without disease 3) tissue sequence correlates, mRNA and protein, should be sought at the cellular level; and (4) sequence-based forms of evidence for causation should be reproducible [18].

Consistent with this model we have provided evidence that BKV is associated with the pathogenesis of HIV-SGD. BKV DNA and gene products were detected in most cases of HIV-SGD and these gene products were not detected in salivary gland tissues from hosts not harboring the disease. Degenerate PCR did not detect herpes viral DNA in HIV-SGD. Nor were herpesviral transcripts detected by viral microarray, or EBV LMP1 or KSHV LANA detected by immunohistochemistry. Lack of Herpesviral protein/transcript detection in HIV-SGD makes etiologic association doubtful. This observation is further supported by the literature. EBV did not have a pathogenic role in HIV-SGD lesions in the pediatric age group [19]. We and others have determined that CMV and HHV-8 were not associated [20]. Counter to the lack of detection of herpes viruses in the disease, one report consistently detected the EBV LMP 1 protein in HIV-SGD, but the cytoplasmic membrane protein was only detected in the nucleus perhaps demonstrating cross reactivity with a nuclear protein [10]. The Rivera study also detected HIV in the ductal epithelium of HIV-SGD which we have confirmed (data not shown). HIV itself does not appear to be sufficient for HIV-SGD development.

The etiologic role for polyomaviruses in human tumors and disease remains debated despite reports that demonstrate polyomavirus nucleic acid and protein [10, 21-22]. In this study, Polyomavirus DNA was detected by degenerate PCR and was confirmed by sequence analysis. While SV40 and JCV DNA were not detected, BK specific primers consistently detected BKV in HIV-SGD. Sequence analysis confirmed BKV's presence. Detection of BK structural and non structural transcripts within diseased tissue coupled with the detection of plasma viremia and oral shedding in the majority of subjects suggested that HIV-SGD was a manifestation of BK productive

infection. Immunofluorescent studies of affected labial minor salivary gland tissue in HIV-SGD consistently demonstrated a nuclear/perinuclear punctuate staining pattern of T antigen with virus specific monoclonal and polyclonal antibodies as previously described [23]. In HIV-SGD p53 co-localized with BKV Tag oncoprotein. Collectively, these results argue that BKV may be an etiologic agent in the pathogenesis of HIV Salivary Gland Disease.

Our results, taken together with the epidemiologic correlates of the disease, have led us to develop a model in which we postulate that during childhood BKV is acquired by primary infection (Figure 2.5). HIV-SGD is closely linked to pediatric HIV with an incidence of 10%-47%⁷[24]. Subsequent to immune suppression a child may develop HIV-SGD and associated morbidity. Healthy children may undergo sub-clinical BKV infection and not clinical salivary gland disease upon primary infection. BKV reactivation, perhaps from the uroepithelium [25], resulting in HIV-SGD. An adult however may experience primary infection or immune suppression-associated and its associated morbidity. The presence of HIV-SGD has been associated with favorable prognosis with regard to HIV disease progression [26]. Thus, we hypothesize that the significant local host immune response and lymphocytic infiltration in diseased salivary glands may imply that the host is capable of mounting a virus specific antigen-driven response. This may indicate that the host is able to respond to other viral infections as well, slowing AIDS progression.

These data support the hypothesis that BK plays a role in the etiology of HIV-SGD. Elucidating a potential etiologic agent and beginning to dissect the pathogenesis of HIV-SGD is the first step toward treatment of this emerging disease.

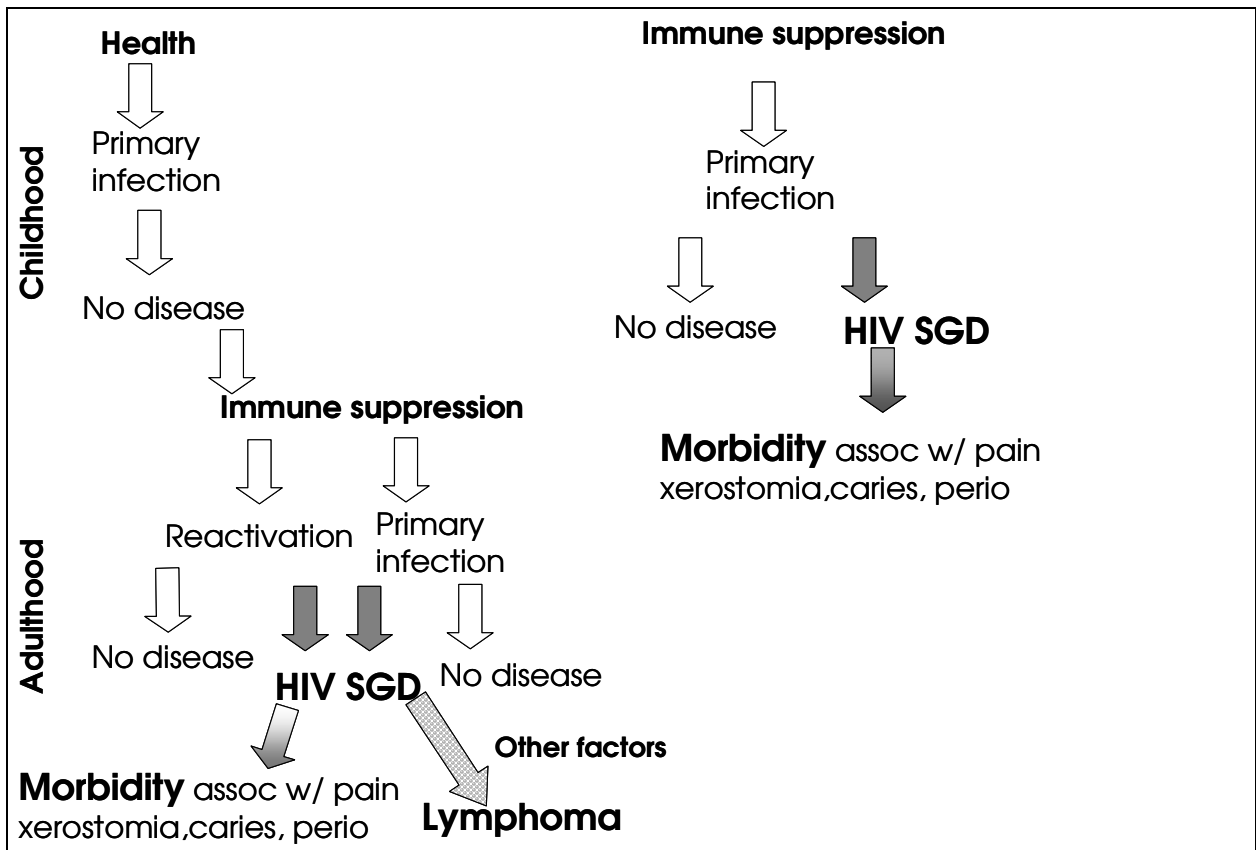


Figure 2.5. Proposed Role for BKV as an infectious agent in the development of HIV-SGD.

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CHAPTER 3

BK VIRUS DETECTED IN SALIVARY GLANDS OF SJOGREN SYMDROME

ABSTRACT

Sjogren's syndrome (SS) is a chronic autoimmune disease characterized by lymphocytic infiltration of the exocrine glands, especially the lacrimal and salivary glands. Although SS was first described in the late 1800's, the etiologic agent is still currently unknown. It has been hypothesized that a persistent viral infection may drive the autoimmune response, however the identity of that virus has remained elusive. The aim of this study is to test the hypothesis that polyomavirus BKV may play a role in SS disease. Minor salivary gland tissue from SS patients and from healthy individual control subjects was examined. Degenerative PCR, sequence analysis and immunostaining were used for virus identification and characterization. BKV polyomavirus DNA was detected in a subset of SS patients and confirmed by sequence analysis. In 2/7 patients clinically diagnosed with SS, BK DNA and protein expression were detected within SS minor salivary gland tissue and not in healthy individuals. A transcript, metastasis associated lung adenocarcinoma transcript 1 (MALAT-1) known to be up-regulated by BKV was also shown to be increased in SS, confirming that BKV may be playing a role in SS disease pathology. We have determined the presence of BKV infection in a subset of SS and report for the first time that BK polyomavirus may be associated with SS disease.

INTRODUCTION

Sjogren's syndrome (SS) is a chronic immune-mediated inflammatory disorder characterized by lymphocytic infiltration of the exocrine glands, especially the lacrimal and salivary glands, and by extraglandular manifestations in approximately 30% of cases. It is a systemic disease, with manifestations from several organ systems such as lungs, kidneys, skin, blood vessels and muscles, and a malignant lymphoproliferative disease may appear in approximately 5–10% of patients. It can occur in a secondary form (sSS) in association with various autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, systemic sclerosis, myositis and others, whereas in the absence of these conditions, the disease is classified as primary SS (pSS).

One of the hallmarks of the syndrome is B cell hyperreactivity that is manifested by hypergammaglobulinemia, circulating immune complexes, and multiple autoantibodies, such as anti-Ro/SSA and anti-La/SSB. Patients with anti-Ro autoantibodies may have a statistically significant increased incidence of parotid swelling, lymphadenopathy, respiratory symptoms and lymphoma [1]. Salivary gland epithelial cells have long been thought to play a central role in the pathophysiology of the syndrome, since they inappropriately express class II major histocompatibility complex, adhesion and functional costimulatory molecules, proinflammatory cytokines, lymphoid chemokines, and autoantigens [2]. Despite better knowledge of the immunopathologic constituents of the syndrome, the etiology of this autoimmune response remains unknown.

It is believed that the etiology of SS consists of genetic, infectious, endocrine and psychoneuroimmunological factors. Several viruses, including EBV, CMV, HCV,

Coxsackievirus and retroviruses have been investigated as important co-factors in the development of SS [1]. However, the data regarding these viruses as a causative agent for SS are contradictory and remains to be established. Recently our group has detected polyomavirus DNA, RNA and protein expression in a related salivary gland disease, human immunodeficiency virus-associated salivary gland disease (HIV-SGD). Using polymerase chain reaction, sequencing and immunohistochemistry techniques, we report for the first time that patients with SS harbor a potential polyomavirus infection in their minor salivary glands (MSG).

Additionally, in previous work performed in our lab we observed BKV-mediated up-regulation of a novel non-coding RNA, MALAT-1 in HIV-SGD. Therefore we investigated the expression of MALAT-1 in SS. Using quantitative real-time PCR we detected up-regulation of MALAT-1 in two SS patients that were consistently positive for BKV DNA and protein expression.

We therefore propose a hypothesis for the role of BKV in the generation of SS autoimmune disorder.

MATERIALS AND METHODS

Patient Sample Collection

Minor salivary gland biopsies were collected from Sjogren's syndrome diagnosed patients from UCSF oral medicine clinic. Tissues were either paraffin embedded or frozen and sectioned for histological analysis. Paraffin-embedded ranula tissue from healthy patients were obtained from the AIDS clinic at UNC hospital in an IRB approved protocol.

DNA Isolation and Polymerase Chain Reaction

DNA extractions were performed for seven SS samples, three ranula samples and BKV-transfected Vero cells at 24 and 48hrs post-transfection using Qiagen DNeasy extraction kit modified for paraffin embedded tissue and Viral DNA extraction kit. Single stranded DNA oligonucleotides were synthesized for PCR primers using a published consensus sequence in the polyomavirus large T antigen gene [3].

Sequencing

Purification of PCR amplicon was carried out with ExoSap-IT (USB) reagent followed by direct sequencing of the large T antigen region using ABI Big Dye® terminator reagents and an ABI PRISM 3730 DNA sequencer (Applied Biosystems, Foster City, CA). Sequences were initially compared using Vector NTI software with EMBL entries for polyoma viruses for BKV (NC_001538), SV40, JCV, WU and KI.

Immunohistochemistry

Sectioned paraffin-embedded SS tissue on slides were used to identify viral proteins in the affected cells. Kidney tissue sections were used as a positive control for BKV infection. Sections were blocked in protein blocking serum, stained with primary antibody targeted to LTag and VP1 then stained with the DAKO LSAB+ peroxidase kit according to manufacturer's specifications. Slides were counterstained with hematoxylin then mounted on coverslips using Permount.

RNA Isolation

RNA was extracted from FFPET using a Paraffin Block RNA Isolation Kit (Ambion) according to manufacturer's instructions. RNA pellets were washed in 75% ethanol and five of each sample pooled in 20 μ L RNA Storage Elution Solution (Ambion). RNA was treated with DNase I using a DNA-free Kit (Ambion), according to the manufacturer's instructions and the resultant RNA used for all further manipulations. RNA was stored at -80°C.

Reverse Transcription

Reverse transcription was performed with SuperScript™ II Reverse Transcriptase Kit. DNase treated RNA was mixed with 1.0 μ L of random hexamer primers(300ng/ μ L), dNTP(10mM) 1 μ L and 9.0 μ L of dH₂O, heated for 5 minutes at 65°C, cooled on ice, and added to 4 μ L 5x first-Strand Buffer and 2 μ L 0.1M DTT, incubate at 25°C for 2 minutes. Add to 1 μ L SuperScript™ II Reverse Transcriptase (RT) (Invitrogen), followed by incubation at 25°C for 10 minutes and denaturation at 72°C for 15 minutes. The resultant cDNA template was stored at -20°C.

Real Time PCR

PCR reactions were performed using an ABI Prism 7000 sequence detection system (Applied Biosystems) and the SYBR Green PCR Core Reagents kit (Applied Biosystems) in accordance to the manufacturer's protocol. Briefly, 2 μ L of diluted cDNA sample was added to 13 μ L of the PCR master-mix. The amplification included a 2-minute 50°C step and an initial denaturation step for 10 minutes at 95°C, followed by 40 cycles consisting

of 15 seconds at 95°C and 1 minute at 60°C. Values were normalized using B-actin to correct minor variations in mRNA.

RESULTS

BKV DNA is detected in a subset of SS

The role of BKV in SS was investigated using PCR specific for the Tag region of BKV. PCR detected BKV DNA in minor salivary glands in 2 of 6 SS patients tested (Figure 3.1, lanes 3 and 6). BKV transfected Vero cells were used as a positive control and non-transfected Vero cells as a negative control (Figure 1, lanes 7,8 and 9). Viral gene products were detected at the expected size, 174 bp and were sequenced in order to confirm the presence of BKV DNA in the disease (Figure 3.2). The region that was amplified was compared to known polyoma genomes and was compatible with the T antigen regions of BK virus with 96-97% homology.

BKV gene expression in a subset of SS

To determine the state of infection within the diseased tissue we examined the expression of viral proteins VP1 and Tag in the SS samples, histologically (Figure 3.3). The absence of viral protein expression would be strong evidence against the role for BKV in the etiology of the disease. Immunohistochemical (IHC) studies were performed that revealed the expression of VP1 and Tag in the acinar epithelial cells of the lesions. Interestingly both proteins were localized to the nucleus. Figure 4 shows sections probed with monoclonal and polyclonal T ag antibodies (rows 1 and 2), VP1 antibody (row 3)

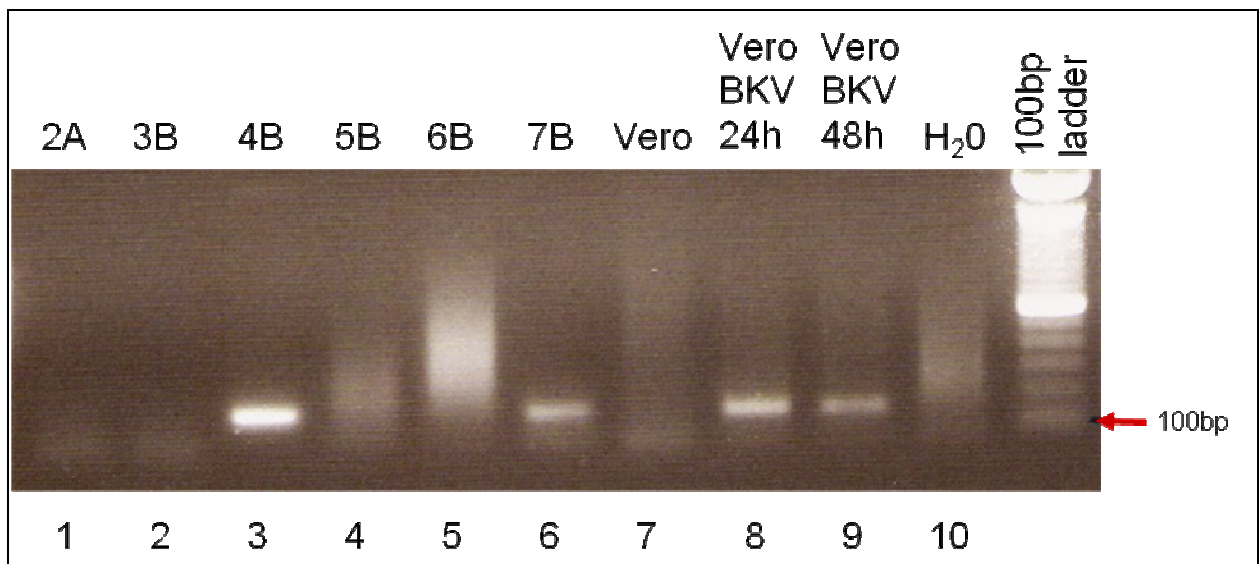


Figure 3.1 BKV DNA detected in a subset of SS patients. PCR amplification of BKV specific T ag DNA in six samples of SS MSGs. Note that patient 4B and 7B SS samples were positive (lanes 3 and 6) and non-transfected Vero cells were negative (lane 7), while BKV-transfected Vero cells were used as positive controls (lanes 8 and 9). Arrow points to 100bp marker.

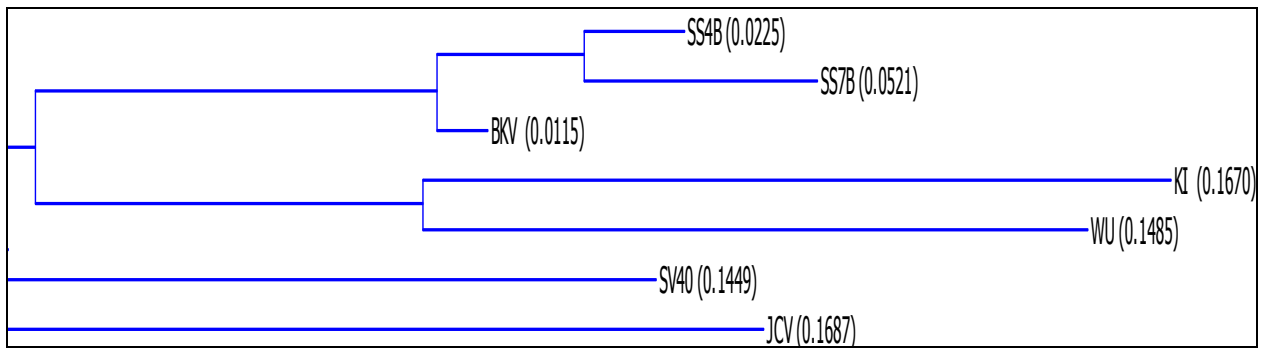
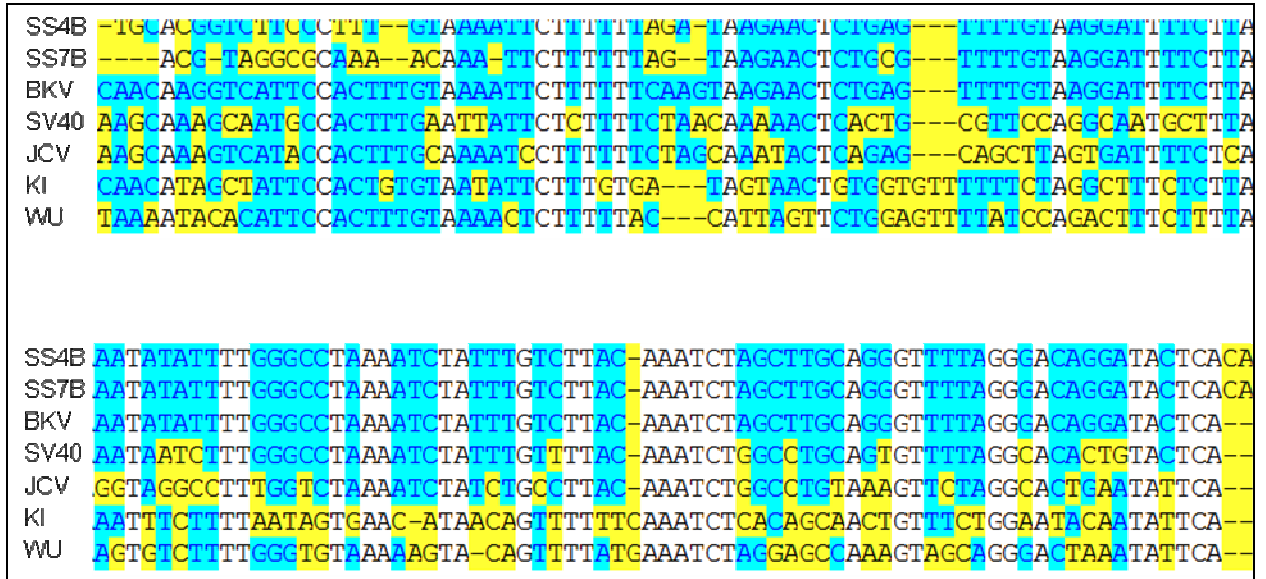


Figure 3.2. Sequence analysis confirmed the detection of BKV DNA in SS. Sequence alignment of SS PCR products from minor salivary gland tissues matched the highly conserved Tag region of BKV but not SV40, KI, WU or JCV. Neighbor joining analysis determined the phylogenetic relationship of SS sequences to BKV.

and isotype matched control antibody (row 4). BKV infected kidney cells were used as a positive control. Arrows point to positive stained cells in kidney cells as well as patients 4B and 7B. No signal was detected in patient 2A. Taken together, the detection of viral DNA and protein expression of non-structural and structural gene products within salivary glands of SS patients suggest that BKV may play an important role in SS disease pathology.

Up-regulation of a novel non-coding RNA, MALAT-1 in a subset of SS

We have previously detected BKV-mediated up-regulation of MALAT-1 in a similar disease, HIV-SGD (unpublished data, Chapter 4). Quantitative real-time RT-PCR was used to measure MALAT-1 levels in biopsied salivary glands of SS patients. The results showed that MALAT-1 was up-regulated five fold in patient SS7B which has been consistently positive for BKV genome and its gene products (Figure 3.4). Levels of mRNA expression is compared to negative control non-transfected Vero cells which is arbitrarily set as 1.

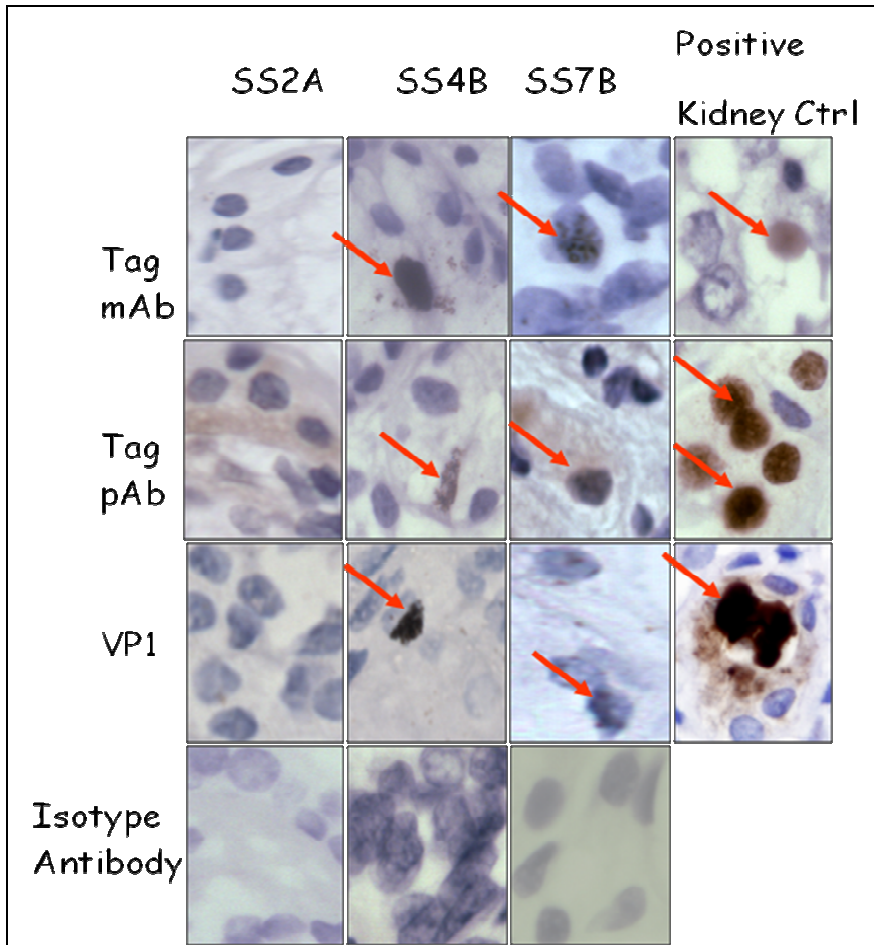


Figure 3.3 BKV gene expression in a subset of SS. Immunohistochemical staining for BKV structural and non-structural proteins. Monoclonal and polyclonal T ag-specific antibodies and polyclonal capsid protein VP1 antibody BKV proteins within the nucleus of minor salivary gland (MSG) epithelial cells in patients 4B and 7B and not in patient 2A with Sjogren's syndrome (SS). BKV-infected kidney cells were used as a positive control. Red arrows indicate BKV positive cells.

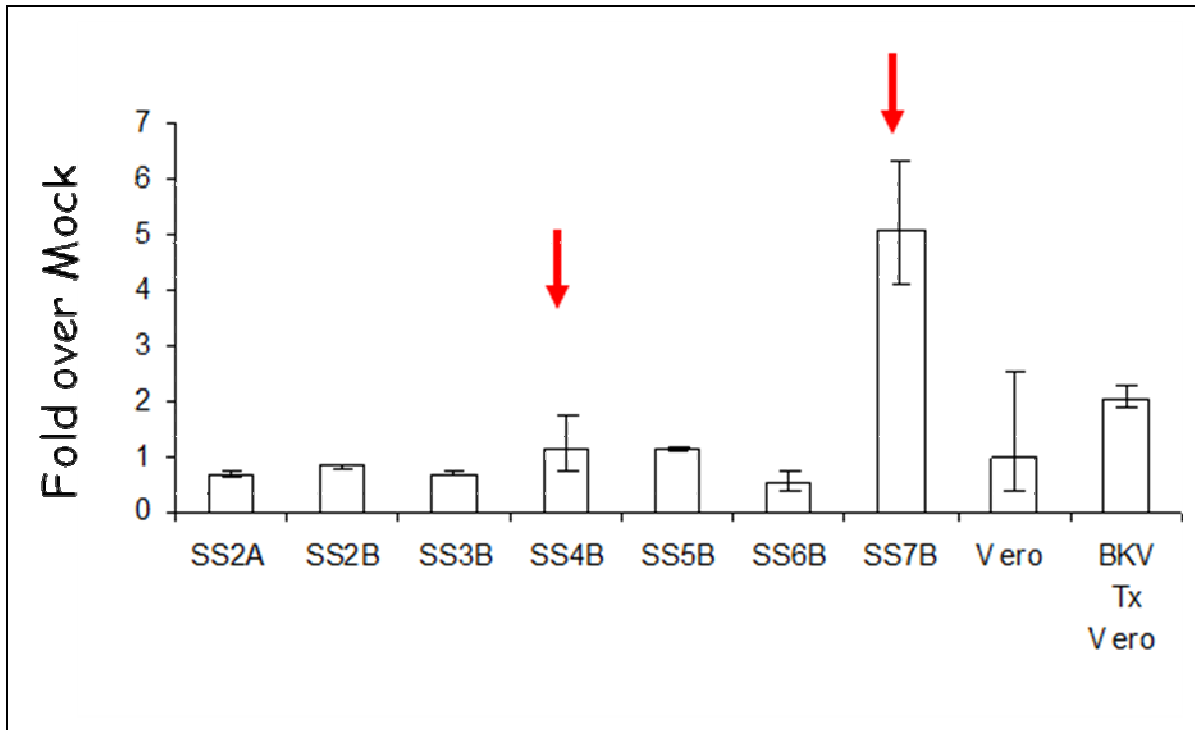


Figure 3.4. Up-regulation of a novel non-coding RNA, MALAT-1 in a subset of SS.

MALAT-1 transcript levels in SS patient MSGs as measured by quantitative real-time RT-PCR was 5 fold higher in patient 7B compared to negative control non-transfected Vero cells which is arbitrarily set to 1, and 2 fold higher than positive control, BKV-transfected Vero cells. Red arrows indicate SS patients positive for BKV DNA and protein.

DISCUSSION

It has long been suggested that many autoimmune diseases represent pathologies induced by various viral agents [4]. However, this association has been fairly vague until now. We report here for the first time that the MSGs of patients with SS are potentially infected with a polyomavirus, BKV. The consistent detection of BKV's genome and non-structural and structural capsid protein in 2 of 7 patients with SS, and none of 3 controls (data not shown) is evidence for an association of BKV infection with SS. Such an association between BKV and SS has not previously been reported.

Amplification products of the Tag from patients 4B and 7B were 96–97% identical to SAU-3 BKV isolate Tag. BKV has previously been linked with autoimmunity, specifically systemic lupus erythematosus (SLE). SLE is the prototype of systemic rheumatic syndrome and is characterized by production of a wide array of autoantibodies. The majority of these autoantibodies are directed against nuclear constituents or antinuclear antibodies (ANA). In patients with SLE, it has been suggested that BKV infection might initiate autoimmunity by inducing antibodies against DNA and histones [5-7]. Using PCR, Sundsfjord et. al[8] identified BKV genomic sequences in 16% of 44 patients with SLE. In another study, 60% of SLE patients showed at least one or several episodes of BKV reactivation while control groups did not have any viral replication[9]. Similarly, several other investigators reported reactivation of polyomavirus in patients with auto-immune diseases including SLE[10-11].

Detection of the main antigenic capsid protein VP1 is evidence against the possibility of persistent latent BKV RNA in the MSGs of patients with SS, while it favors the presence of potentially actively replicating BKV. The hapten-carrier model is most

commonly used to describe how viruses can induce autoimmune diseases such as SS or SLE. Viral proteins can form complexes with cellular chromatin and when released outside of the cell either upon virus-induced cell lysis or killed by CD8⁺ T cells, becomes bound by DNA-specific B cells which subsequently process and present peptides derived from both the viral protein and for example histones. This may be sufficient to transform B cells to anti-DNA antibody-producing plasma cells and to terminate histone-specific T cell anergy. Termination of T cell anergy may occur as a result of activation of virus-specific CD4⁺T cells which start to secrete IL-2 into the microenvironment. IL-2 may then induce proliferation of histone-specific T cells which may terminate the anergic state of these autoimmune T cells.

Previous work from our lab has determined that BKV infection of patient MSGs and salivary gland cells in vitro result in up-regulation of a novel non-coding RNA, MALAT-1 (unpublished data, Chapter 4). Similarly, SS patients that were positive for MSG BKV infection also showed up-regulation of MALAT-1. MALAT-1 is widely expressed in normal tissue and while little is known about its function, appears to be a potentially generic marker for epithelial carcinomas [12-17]. MALAT-1 has been found to be up-regulated in lung adenocarcinoma metastasis [13], endometrial stromal sarcoma of the uterus [15] and non-hepatic human carcinomas [14]. MALAT-1 up-regulation has also been shown to be a predictor of unfavorable osteosarcoma drug therapy outcomes [12]. This initial description of MALAT-1 up-regulation by BKV in SS is of significant interest and importance because there is evidence that SS patients have a higher risk of developing malignancies such as cervical, lung and breast cancer [18, 19 ,reviewed in, 20]. However, further work needs to be done to ascertain not only the precise underlying

mechanisms behind the increased risk of cancer in SS but also the significance of increased MALAT-1 expression in SS.

Although great advances have been made over the past years in understanding the mechanism of SS disease to provide better therapies, their etiologies are largely unknown. We report here for the first time an association of BKV infection with SS, a finding that may lead to development of novel therapeutic strategies for autoimmune disorders.

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CHAPTER 4

BK VIRUS UP-REGULATES A NOVEL NON-CODING RNA ASSOCIATED WITH METASTASIS, MALAT-1

ABSTRACT

In this study it was determined that BK virus (BKV) oncoproteins up-regulate the novel non-coding RNA, metastasis-associated lung adenocarcinoma transcript-1 (MALAT-1). While MALAT-1 is consistently up-regulated in several epithelial malignancies, little is known about its function or regulation. MALAT-1 up-regulation was detected by suppressive subtractive hybridization (SSH) and quantitative real-time RT-PCR of BKV-infected minor salivary gland biopsies from HIV infected subjects with salivary gland disease (HIV-SGD). In a transgenic mouse model of salivary gland disease that expressed the homologous BKV oncoprotein, mouse polyoma middle T antigen, an increase in MALAT-1 transcription was detected in transgenic mice compared to wild type mice. Both wild type BKV infection and over-expression of BKV T antigen (both LT and ST) in vitro confirmed that these oncoproteins were responsible for up-regulating MALAT-1. These viral proteins functioned by increasing the activity of the MALAT-1 promoter region. In conclusion, we have determined that BKV infection plays an important role in the regulation of MALAT-1 transcription. This study details the up-regulation of a malignancy associated transcript by a DNA tumor virus and is the first report of a mechanism for MALAT-1 regulation.

INTRODUCTION

BK virus (BKV) belongs to the polyomavirus family and is ubiquitous in the human population [1]. The viral genome consists of a closed circular double-stranded DNA molecule of approximately 5 kb encoding an early region, a late region and a regulatory region (RR) containing promoters, enhancers and the replication origin. The genome is transcribed bi-directionally from the origin, with the early region encoding for two non-structural proteins: large tumor antigen (LTag), and small tumor antigen (STag). The late region encodes for structural capsid proteins VP1, VP2 and VP3 and the non-structural agnoprotein [1]. Transformation and oncogenicity induced by BKV is attributable to the viral oncoproteins, LTag and STag. LTag acts mainly by blocking p53 and pRB family tumor suppressor protein functions and by inducing host cell chromosomal aberrations and instability [1]. The principal effect of STag is to bind the catalytic and regulatory subunits of the protein phosphatase PP2A, thereby constitutively activating the β -catenin pathway which drives cells into proliferation [1]. The mouse polyoma virus middle T antigen (MT ag) is functionally homologous to BKV ST ag and its over-expression results in salivary gland enlargement and pathology [1, Maglione, 2001 #184].

BKV DNA has been isolated in several human tumors, both integrated into the genome and episomally. Sites include bone, pancreatic islet cells, kidney, urinary tract, prostate and a wide variety of brain tissues [2-9]. Most recently our group consistently detected BKV within salivary gland tissues of HIV infected subjects and have determined that salivary gland cells are capable of productive BKV infection in vitro (Jeffers et al in press, Chapter 2, unpublished data).

Metastasis-associated lung adenocarcinoma transcript 1 (MALAT-1) is a novel non-coding RNA. The MALAT-1 gene, also known as α gene, is found on chromosome 11q13 and is well conserved among the mammalian species [10]. The MALAT-1 transcript has been shown to localize to the nucleus [11] and currently has no known function except that its 3' end can be processed to yield a tRNA-like cytoplasmic RNA [12]. MALAT-1 is widely expressed in normal tissue and while little is known about its function, appears to be a potentially generic marker for epithelial carcinomas [10, 13-17]. MALAT-1 has been found to be up-regulated in lung adenocarcinoma metastasis [10], endometrial stromal sarcoma of the uterus [15] and non-hepatic human carcinomas [14]. MALAT-1 up-regulation has also been shown to be a predictor of unfavorable osteosarcoma drug therapy outcomes [13].

In this study, use of suppressive subtractive hybridization (SSH) demonstrated significant differential expression of MALAT-1 in HIV positive patients with salivary gland disease (HIV-SGD). Further, MALAT-1 was up-regulated in a MTag transgenic mouse model of salivary gland disorder, in an in vitro model of wt BKV infection, and in BKV LT and STag expressing cells. Finally, we provide strong evidence that the MALAT-1 promoter activity was up-regulated upon BKV oncoprotein expression, in vitro. Collectively, these data suggest a role for LT and ST antigen-mediated up-regulation of MALAT-1.

Gene	Description	Classification	Library	
MALAT1	metastasis associated lung adenocarcinoma transcript 1 (non-coding RNA)	unknown	Plus	4
HLA-B	major histocompatibility complex, class I, B		Plus	2
PIGR	polymeric immunoglobulin receptor	immunity/defense	Plus	3
CYTB	cytochrome b	transport /mitoch	Plus	3
PIP	prolactin-induced protein	inhibition T lymphocyte apoptosis	Plus	2
RPLP0	RPLP0		Plus	2

Table 4. Genes up-regulated in HIV-SGD using suppressive subtractive hybridization.

MATERIAL AND METHODS

Subjects, animals and cell culture

Minor salivary glands (MSG) were dissected from the lower lips of 10 HIV-positive patients with SGD and from 7 healthy control subjects (with or without ranula) in IRB approved protocol at either the UNC University Hospital dental clinic or UCSF Oral AIDS Center. Biopsy samples were snap-frozen and kept in liquid nitrogen or formalin-fixed and embedded in paraffin until the RNA extraction procedure.

Four MMTV/PyV-mT transgenic mice and four wild type mice parotid samples were kindly donated from Dr. Lesley Ellies.

HSY cells are a neoplastic epithelial cell line initially established in culture from a human parotid gland adenocarcinoma [18]. HSY cells were obtained as a gift from Dr. B. Baum (NIH) and cultured in Dulbecco's minimal essential medium (DMEM; Sigma). African monkey kidney cells or Vero cells (American Type Culture Collection [ATCC]) were cultured in DMEM (Sigma). All cell types were grown in medium supplemented with 10% fetal bovine serum (FBS) (Sigma), and 1% penicillin-streptomycin (pen/strep)(Gibco) unless otherwise stated and maintained in a humidified 37⁰C, 5%CO₂ chamber.

Suppression Subtractive Hybridization (SSH)

One to two µg of pooled biopsied salivary glands from HIV-SGD patients and pooled control (healthy persons) RNA was sent to Evrogen (Russian Republic) for SSH. In brief, SMART technology was used to synthesize cDNA, which was subsequently digested with RsaI in preparation for subtraction in both directions (HIV-SGD as

driver/control as tester, and vice versa). Adaptors were ligated to the two cDNA populations, followed by two rounds of hybridization and amplification. The secondary PCR products from the two subtracted populations were then ligated into the pAL9 vector.

Sequencing of subtracted cDNA library

Two hundred and fifty positive clones from each of the subtractions were randomly selected and cultured in 1.5 mL LB-medium in duplicate 96-well plates at 37 °C overnight. Clones were selected by blue-white screening (Promega) and sequenced using a M13 forward promoter (-21) primer (5'-TGT AAA ACG ACG GCC AGT-3') and BigDye 3.0 sequencing mix (Applied Biosystems) before analysis by capillary electrophoresis on an ABI 3700 genetic analyser (Applied Biosystems). The sequences of the inserts of differentially expressed genes were identified using NCBI Blast search (blastn).

RNA extraction and cDNA synthesis

Total RNA was extracted from pooled MSGs of HIV-SGD patients, pooled MSGs of healthy controls, mouse parotid frozen tissues and Vero cells using RNeasy MiniKit (Qiagen, USA). Total RNA isolation from formalin fixed tissue using the Optimum FFPE RNA Isolation Kit (Ambion Diagnostics, INC) according to manufacturer's instructions. The RNA was suspended in nuclease-free water and quantitated by UV spectrophotometry, aliquoted and stored at -80°C. One µg total RNA from the HIV-SGD and control tissues, mice parotid tissue and Vero cells were reverse transcribed to cDNA

using random primers and the SuperScript[™] II Reverse Transcriptase (RT) Kit (Invitrogen) as described by the manufacturer. Contaminating DNAs were removed by use of RQ1 DNase kit (Promega) as described by the manufacturer.

Northern Blot

Total RNA was isolated using TRizol (Invitrogen) as described by the manufacturer. Contaminating DNAs were removed by use of RQ1 DNase kit (Promega) as described by the manufacturer. 10 ug of RNA was subjected to electrophoresis and Northern blotting. MALAT-1 was hybridized with a ³²P-labeled oligonucleotide probe for MALAT-1

Semi-quantitative and quantitative real-time RT-PCR

Semi-quantitative RT-PCR was performed using previously published primers for Tag [19] and Taq Polymerase (Qiagen, USA). The following program was used for amplification: 95°C for 2 min (1x); 94°C for 45 sec, 56°C for 45 sec, 72°C for 30 sec with 2 sec increase per cycle (35x), 72°C for 10 min (1x). Amplified cDNA was electrophoresed on 2% agarose gel (Sigma).

Real time RT-PCR was performed with LightCycler 480 Syber Green I Master Mix in the presence of transcribed cDNA and 0.25mM of gene specific primers. Experiments were performed in duplicate and β -actin was used as an internal control. Duplicate or triplicate Ct values were averaged, normalized to an average β -actin Ct value, and fold activation in healthy vs diseased tissue was calculated using the $2^{-\Delta\Delta Ct}$

method. For Vero cells, gene expression values are presented as the changes (*n*-fold) in T Ag transcript levels, with the levels in non-transfected/mock samples arbitrarily set to 1.

Immunofluorescence/Immunohistochemistry

Frozen sections were cut from minor salivary glands (HIV-SGD and control glands) and from mouse parotid glands (transgenic and wild type) for immunofluorescent and confocal analysis. Tissue sections were fixed, blocked, then stained with either PAb416 (Genetex) antibody specific for SV40 T antigen or IgG isotype control. PAb416 has been shown to cross react with BKV Tag and is commonly used for BKV Tag detection. Both antibodies were incubated with fixed cells for 1 hr at 37⁰C followed by a fluorescein-conjugated anti-mouse (Sigma) antibody (1:20). Slides were overlaid with Vectashield (Vector Laboratories) then subjected to confocal microscopy.

Formalin fixed sections were deparaffinized, and washed. Slides were incubated in 3% hydrogen peroxide and blocked and incubated with PAb416 (Genetex). DAKO LSAB+ peroxidase kit (DAKO Corporation) was used according to manufacturer's specifications.

Plasmid cloning, transfection and infection

A 5.5 kb region upstream of MALAT-1 transcription site was amplified using primers 5'GGGACGCGTAAAGAGGATTCTATCTAACAAGGA3' and 5'GGGCTCGAGGAAACGTGAAAACCCACTCT3' and inserted into pSeap2-Basic expression cloning vector (Clontech) using restriction sites MluI and XhoI. To determine MALAT-1 promoter activity, Vero cells were first infected with BKV as previously

described (Jeffers et al, 2009, in press) with 64 HAU of virus for 24 h. At 24 h post infection (hpi), virus was removed from the culture media, cells were washed with 1X PBS and replaced with fresh medium containing the MALAT-1 promoter plasmid and β -gal construct plus transfection reagent. At stated times post transfection the cell monolayers or supernatant were collected for immunoblot assays, beta-galactosidase enzyme assay (Promega) or secreted alkaline phosphatase enzyme assay (Promega) according to manufacturers instructions.

For MALAT-1 transcript evaluation in vivo, Vero cells were transfected with pBKV35-1 plasmid DNA (ATCC 45026, MD). Following incubation at 37 °C for 24 h, 48 h, or 72 h both untreated (mock) and transfected cells were harvested for RNA extraction and RT-PCR.

Immunoblotting

Total cell protein was extracted using 1% SDS lysis buffer (1% (w/v) SDS, 0.05M Tris.Cl pH8, 1mM DTT). Protein concentrations were determined using the BioRad protein assay, and equal amounts of protein were electrophoresed on a 10% Bis-Tris polyacrylamide minigel (Invitrogen). PAb416 (1:200) (Genetex) in 5% non-fat dry milk in 0.1% Tween-20 PBS (PBS-T) was used to detect T Ag expression and Actin (C-11)-R sc-1615-R (1:1000)(Santa Cruz Biotechnology) in 1% BSA/TBS-T for actin expression. After washing in PBS-T/TBS-T, blots were probed with a horseradish peroxidase-conjugated secondary antibody (1:10,000) (Promega). Antibody complexes were detected using SuperSignal West Pico Chemiluminescent substrate (Thermo scientific) and exposed to film (Kodak).

Beta galactosidase enzyme assay

β -Galactosidase Enzyme Assay System with Reporter Lysis Buffer (Promega) was performed according to manufacturer's instructions. Briefly, cells were collected, centrifuged and resuspended in 1X lysis buffer and transferred in triplicate to 96 well plates. Diluted sample was added to an equal volume of Assay 2X Buffer, which contained the substrate ONPG (o-nitrophenyl-beta-D-galactopyranoside). Samples were incubated for 30 minutes, terminated by addition of sodium carbonate, and the absorbance read at 420nm with a spectrophotometer.

Secreted alkaline phosphatase reporter gene assay, chemiluminescent

SEAP assay was performed according to manufacturer's instructions. Briefly, culture supernatant from transfected or mock (untreated) cells were collected at stated times post-transfection. Collected sample was diluted 1:4 in Dilution buffer, heated at 65°C for 30 min then centrifuged at maximum speed to 30 s. Heat-treated samples were then transferred to a microplate (black or white), inactivated with Inactivation Buffer then treated with Substrate Reagent. Chemiluminescence was measured using a luminometer.

RESULTS

Differential gene expression in HIV-SGD by SSH

Differential gene expression in minor salivary glands from patients with HIV-SGD and healthy control subjects was examined with the use of SSH. Pooled RNA from ten HIV-SGD positive subjects and from seven HIV negative subjects was utilized to diminish individual variation. Two SSH libraries were constructed, disease (plus) and healthy library (minus). The minus library was enriched for genes whose expression was suppressed in HIV-SGD and the plus library was enriched for genes whose expression was induced by disease development. 250 randomly selected clones were sequenced from the minus SSH library and 250 clones from the plus library; of those 232 and 208 contained cDNA inserts, respectively. The SSH library was automatically processed using a specifically created software tool. This processing included 3 basic steps: finding adaptor sequences on the 5' and 3' ends of a clone as markers and extracting a fragment from the clone, blasting the batch of fragments, clustering alignments and linking with unique gene identification. We were able to align 436 sequences with known or predicted human genes. Our total number of alignments reached 502 due to 57 chimeric clones which had 2 or three clones from different RNA's linked together during SSH. Of the 436 cDNAs 73 sequences were identified that did not match to any known or predicted mRNAs but aligned with the human genomic DNA and with EST databases. These were considered novel.

A non redundant set of the genes was created which excluded highly expressed clones present in both disease and healthy genes in the plus library. There were 6 genes found more than once among sequenced SSH clones in each of the libraries (Table 1). It

was shown previously that a number of cDNA fragments corresponding to a gene in the SSH library correlates with a degree of differential expression of the gene [20].

Considering all of the differentially expressed clones detected by SSH analysis, major functional classes of transcriptionally regulated genes in minus library included signal transduction (9%), cell cycle(8%), immune/defense(8%), synthesis/ development/ differentiation (8%) and transport (8%). Transcriptionally regulated genes in the plus expression library included those involved in signal transduction (15%), synthesis/development/differentiation(13%), apoptosis(8%), metabolism(8%) and transport. The largest category of de-regulated genes were unknown, among these was the metastasis associated lung adenocarcinoma transcript-1 (MALAT-1).

MALAT-1 is up-regulated in Salivary Gland Disease

To confirm the SSH data, quantitative real-time RT-PCR was performed using primers specific for MALAT-1. A 2.6 fold increase in MALAT-1 was detected in HIV-SGD positive tissue compared to non-diseased tissue. Results were normalized to the levels of β -actin, using the $2^{-\Delta\Delta C(T)}$ method, and are presented as the changes (*n*-fold) in MALAT-1 transcript levels. Non-diseased tissue samples were arbitrarily set to 1.

A transgenic mouse model of salivary gland disease (SGD) expressing mouse polyoma middle T antigen displays clinically observed salivary gland enlargement similar to HIV-SGD [21]. Mouse polyoma middle T antigen shares functional homology with small t antigen of the human BKV by complexing with c-src and pp2A [1]. Semi-quantitative RT-PCR detected amplification of MALAT-1 cDNA from 4/4 transgenic

mice with SGD while the transcript was consistently detected at much lower levels in 4/4 wild type mice (Figure 4.1B).

BKV is detected in HIV-SGD

We have previously shown BKV consistently detected in HIV-SGD salivary gland biopsies (Chapter 2, unpublished data). Further, BKV DNA was significantly increased in HIV-SGD patient serum and saliva (Chapter 2, unpublished data, Jeffers et al in press). BKV Tag was detected within biopsied salivary glands of HIV-SGD subjects by immunofluorescence (representative image shown Figure 4.2A).

Immunofluorescent studies of affected labial minor salivary gland tissue consistently demonstrated a nuclear/perinuclear punctuate staining pattern of T antigen with virus specific antibodies as previously described in prostate cancer [9, 22].

Immunohistochemical stains of paraffin embedded tissues consistently detect BKV Tag using a polyomavirus specific monoclonal antibody (Figure 4.2B).

BKV up-regulates MALAT-1 in vitro

Infection of a parotid salivary gland cell line, HSY, with BKV resulted in increased MALAT-1 expression by quantitative real-time RT-PCR (Figure 4.3A). An increase in the 8.1 KB MALAT-1 transcript was detected in another BKV permissive cell line, Vero cells, by Northern blot analysis. U6 RNA was also probed as a loading control (Figure 4.3B). To determine whether BKV Tag expression modulated MALAT-1 transcription, both LT and ST antigen oncoproteins were over-expressed in Vero cells. Both semi-quantitative RT-PCR (Figure 4.3C) and quantitative real-time RT-PCR were

A.

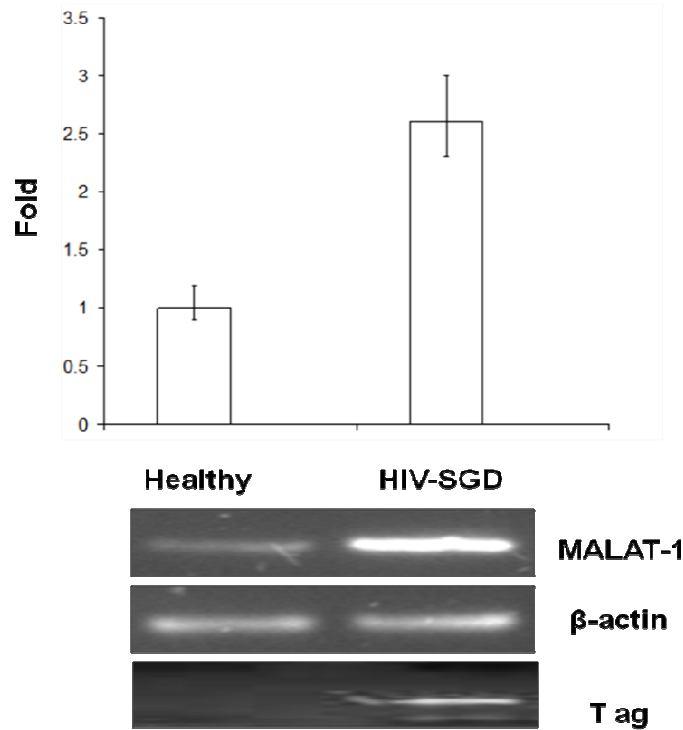


Figure 4.1. Up-regulation of MALAT-1 in salivary gland disease. MALAT-1 transcript levels are elevated in salivary gland biopsies from **A.** HIV-SGD patients and **B.** an SGD mouse model. **A.** MALAT-1 transcript levels in human minor salivary gland biopsies were measured by quantitative real-time RT-PCR and found to be significantly higher in subjects diagnosed with HIV-SGD compared to healthy subjects without the disease. MALAT-1 amplified cDNA is shown for healthy (left panel) and diseased samples (right panel) in comparison to internal control, β -actin amplified cDNA.

B.

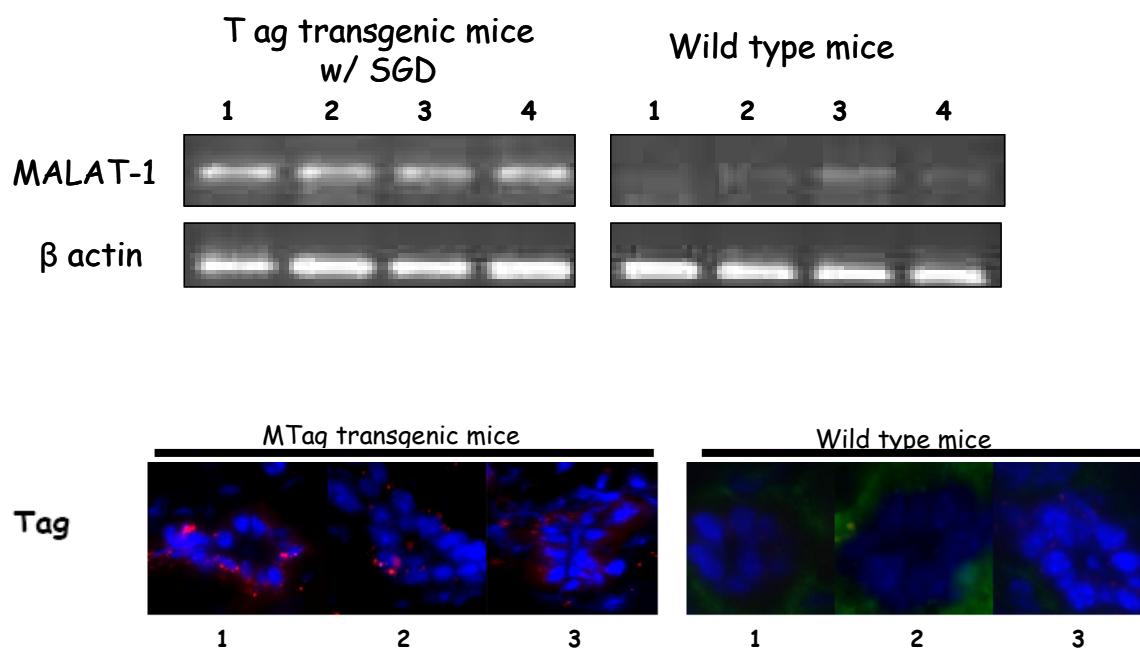
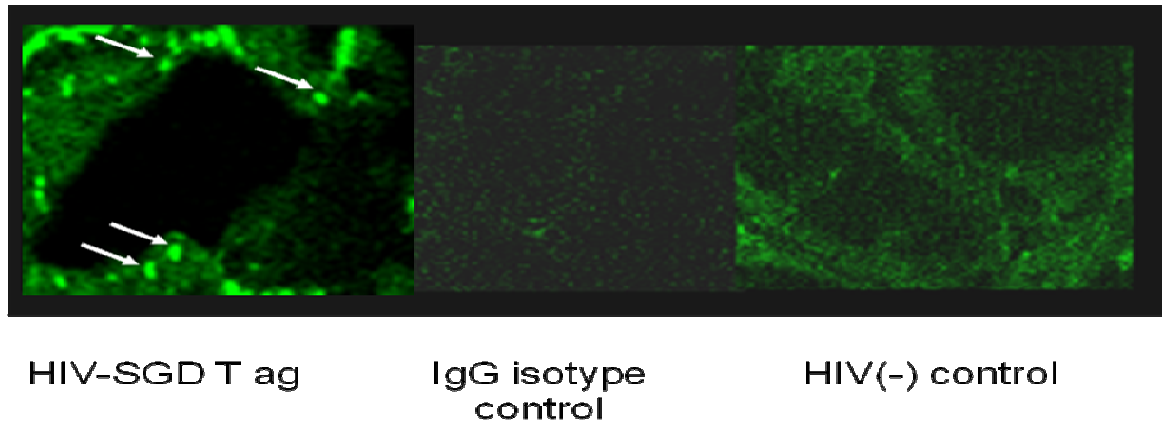


Figure 4.1 (continued) B. MALAT-1 transcript levels in mouse salivary gland biopsies were measured by semi-quantitative RT-PCR and found to be significantly higher in middle T antigen transgenic mice with SGD compared to wild type mice without the disease. MALAT-1 was amplified from a cDNA library of mouse SGD, shown are four diseased (left panel) and four wild type (right panel) mice compared to internal control, β -actin amplified cDNA.

A.



B.

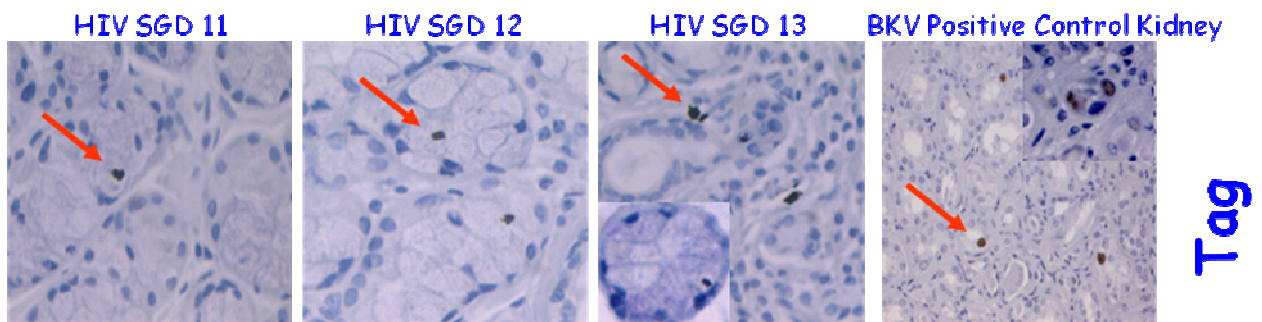


Figure 4.2. BKV detected in salivary gland biopsies of HIV-SGD patients. (A)

Immunofluorescent staining showing BKV T ag protein (green) expression within salivary gland cells of HIV-SGD patients (left), IgG isotype control (middle) and HIV(-) patients (right). **(B)** Immunohistochemical staining of HIV-SGD salivary gland biopsies showing nuclear BKV T ag (arrows) expression and BKV infected kidney cells (right) as a positive control.

performed (Figure 4.3D). At 24 h and 48 h post-transfection an increase in MALAT-1 transcript levels was observed using both techniques. By quantitative real-time RT-PCR a 2.3 and 5.5 fold increase in MALAT-1 transcripts was detected compared to non-transfected/mock Vero cells. Results were normalized to the levels of β -actin, using the $2^{-\Delta\Delta C(T)}$ method, and are presented as the changes (*n*-fold) in MALAT-1 transcript levels. Mock samples were arbitrarily set to 1 (Figure 4.3D).

BKV infection upregulates MALAT-1 promoter

Upon determination that the MALAT-1 transcript was up-regulated in the presence of BKV Tag in vivo and in vitro, the effect of BKV oncoprotein expression on MALAT-1 promoter activity was investigated in the context of the whole virus. Examination of the enhancer/promoter region 5.5 kb upstream of the ATG start site using the PROMO 3.0, prediction of transcription sites program, detected many potential binding sites. The similarity of the sequence to the transcription binding site is greater than 98% (or dissimilarity of less than 2%). Interestingly, numerous consensus binding sites were detected upstream of MALAT-1 start site consistent with the previously described activities of BK viral oncoproteins [23-25] including five p53 binding sites (green), three T antigen binding sites (red), two TCF-2 sites (purple) and two E2F-1 sites (blue) (Figure 4.3A). As described in the methods, 5.5 kb upstream of the MALAT-1 coding region was amplified and cloned into a pSEAP expression vector. BKV infected Vero cells were transfected with the MALAT-1 promoter reporter construct. Promoter activity was up-regulated 7 fold at 72 hours post-transfection of the cloned MALAT-1 promoter. BKV T antigen expression within

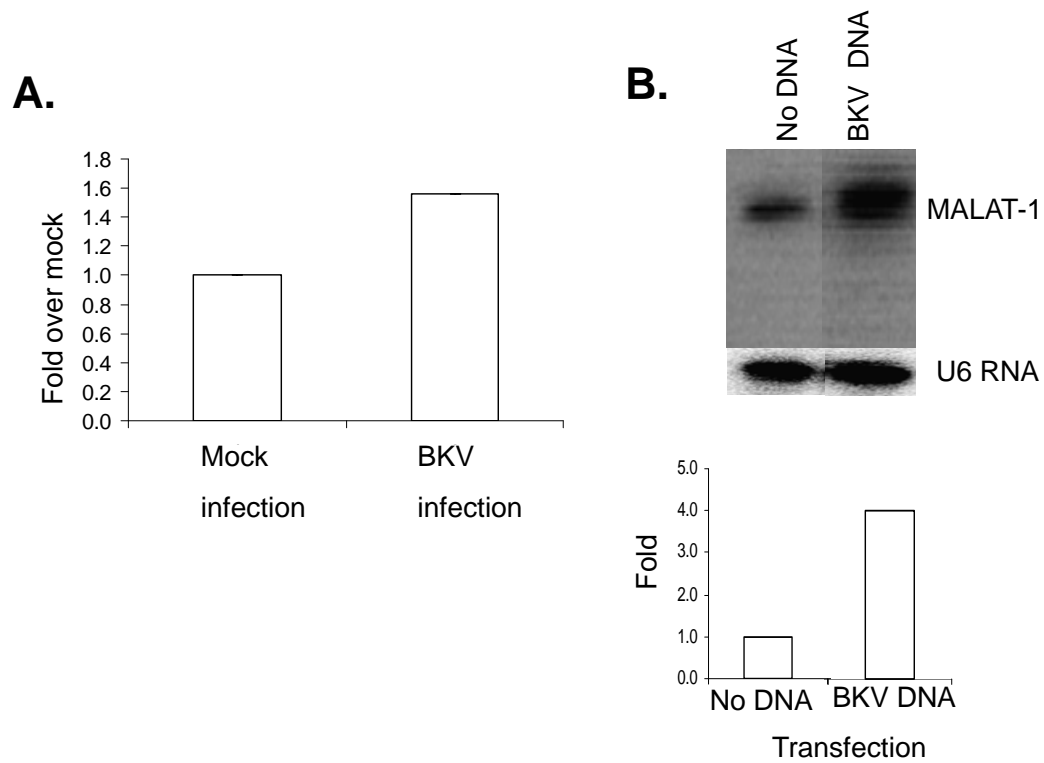
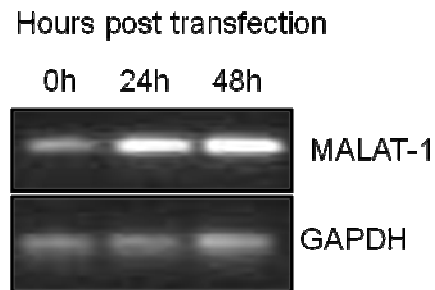


Figure 4.3. BK virus up-regulates MALAT-1 transcript in vitro. (A) MALAT-1 transcript levels in HSY cells as measured by quantitative real-time RT-PCR was 1.6 fold higher in BKV infected cells compared to uninfected/mock at four days post infection. (B) Northern blot analysis showing four fold increase in MALAT-1 transcript in Vero cells transfected with BKV DNA.

C.



D.

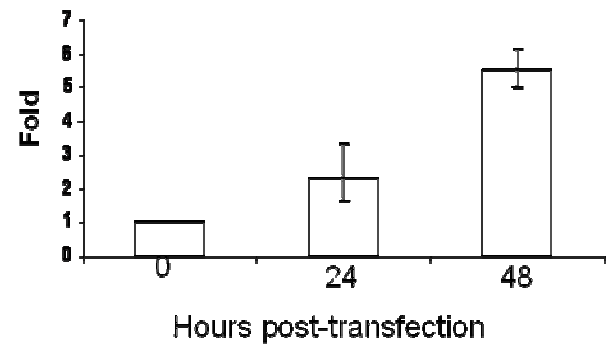


Figure 4.3. (continued). C) Amplified cDNA bands showing an increase in MALAT-1 transcript in Vero cells expressing BKV LT/ST antigen compared to untransfected/mock. (D) MALAT-1 transcript levels in Vero cells as measured by quantitative real-time RT-PCR was three and six fold higher at 24 h and 48h post transfection.

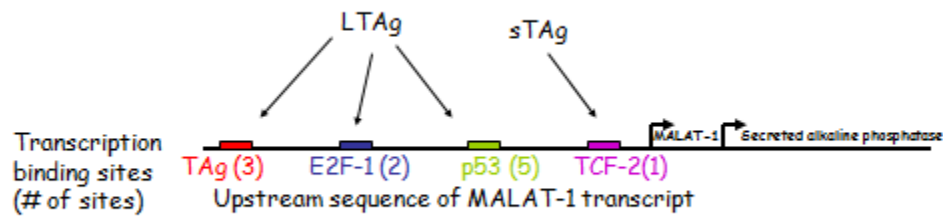
these cells was confirmed by immunoblot assay using a polyomavirus T ag specific monoclonal antibody (Figure 4.4B).

DISCUSSION

In this study we have shown that a novel non-coding RNA, MALAT-1 is up-regulated by the human polyomavirus, BKV. While BKV is a known DNA tumor virus and has been implicated in a number of pathologies [2, 8-9, 19, 26-28], little is known with regard to BKVs modulation of host gene expression in disease. However, differential gene expression studies described here determined that MALAT-1 was the most differentially expressed transcript in a BKV-associated salivary gland pathology, HIV-SGD.

In a survey of normal tissues it was determined that MALAT-1 was expressed in numerous cell types, with highest relative expression in the normal pancreas and lung, 2.0 and 1.6 fold respectively [10]. MALAT-1 is associated with epithelial cell malignancies including non-small cell lung tumors, stromal sarcoma and non-hepatic carcinomas. MALAT-1 expression is associated with a five-fold increased risk of subsequent metastasis in non-small cell lung tumors compared to low expressing tumors. In fact, in non-small cell lung carcinoma, MALAT-1 expression is an independent prognostic parameter for survival [10]. Further, high expression of MALAT-1 is also a negative predictor for osteosarcoma drug therapy outcome [13]. We show that MALAT-1 is up-regulated in another epithelial disease, a BKV

A.



B.

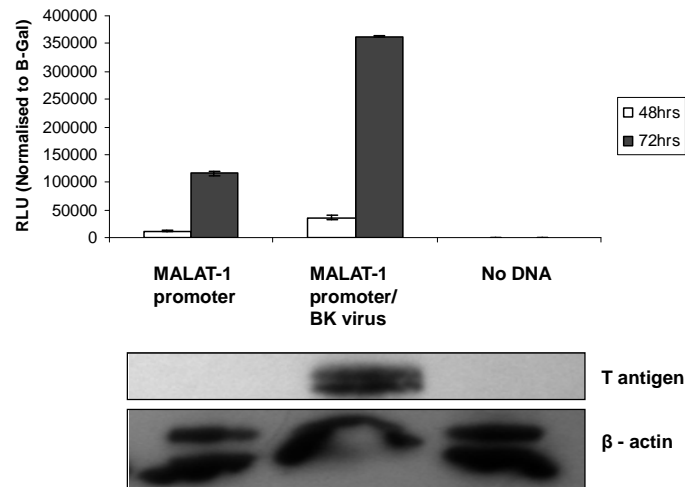


Figure 4.4. BKV infection up-regulates MALAT-1 promoter activity. **A.** 5.5 kb MALAT-1 promoter region (black) showing T antigen (red), p53 (green), TCF-2 (purple) and E2F-1 (dark blue) binding sites. MALAT-1 coding region is shown in light blue. **B.** MALAT-1 promoter activity was significantly increased in the presence of BK viral infection at 48 and 72 h post-transfection. Immunoblot showing BKV T antigen protein expression in cells infected with BKV. Antibodies against T Ag (top panel), β-actin (bottom panel) were used.

associated salivary gland pathology, HIV-SGD. This initial description of MALAT-1 up-regulation by this small DNA tumor virus is of significant interest and importance.

The success of BKV and its related polyomaviruses as formidable DNA tumor viruses is attributable to expression of their T antigen oncoproteins. The ability of LT and ST antigens encoded by human polyomaviruses to ablate p53 and pRb function and enhance pp2A signal transduction are well documented [23, 25, 29]. Mouse Polyoma MT antigen shares 79 amino acids that represent a DnaJ domain with both BKV LT and ST, as well as an additional 112 amino acids with BKV ST antigen[1]. Almost all of the sequence of BKV STag is contained within the MTag of mouse polyoma virus and both the mouse and human antigens form complexes with protein phosphatase 2A (pp2A) [30-31].

HIV-SGD is a polyomavirus-associated salivary gland pathology, in which BKV Tag was detected within acinar and ductal cells of the salivary gland (unpublished data, Chapter 2, Fig 2). MALAT-1 up-regulation was consistently detected in HIV-SGD. To further confirm Tag-mediated MALAT-1 up-regulation in vivo, a mouse model of polyomavirus associated salivary gland disease was explored. Mice infected with polyomaviruses not only develop salivary gland disease but often go on to develop malignancies within the glands [21, 32]. A mouse polyoma middle T antigen-expressing transgenic model with SGD pathology was used [21]. Interestingly, MALAT-1 was highly expressed in transgenic animals expressing the functional homologue of the BKV oncoprotein compared to wild type animals.

In vitro studies were also performed to confirm BKV's role in MALAT-1 up-regulation. Parotid gland cell lines were infected with BKV in vitro and transcript levels

monitored. At four days post infection relative MALAT-1 transcript levels were 1.6 fold as compared to uninfected/mock cells (Figure 3A). Considering that Ji et al, determined normal salivary gland cells express negligible MALAT-1 and that comparisons of MALAT-1 expression in non-metastatic and metastatic lung tissues reveal a 3 fold difference in expression levels[10], this 1.6 fold difference may potentially be biologically significant. Increased MALAT-1 transcripts were also consistently detected by quantitative real-time RT-PCR in HSY cells that were permissively infected with BKV (Figure 4.3A) or in Vero cells that over-expressed LT/ST antigen by Northern blot (Figure 4.3B), quantitative (Figure 4.3D) and semi-quantitative real time RT-PCR (Figure 4.3C). The implication of the onco-RNA's association with a DNA tumorvirus is significant.

MALAT-1 has been consistently up-regulated in several epithelial malignancies hence its expression and detection are of significant biologic importance. In an initial step toward understanding how BKV regulates MALAT-1, a reporter assay was developed to assay activation of MALAT-1's enhancer/promoter region. Upon BKV infection increased reporter activity was observed. Several potential binding sites for BKV Tag were detected within the enhancer/promoter region as well as p53 binding sites, TCF-2 and E2F-1 sites. Further work would need to be done to determine which region within the 5.5kb length upstream of MALAT-1 binds specifically to BKV oncoproteins.

In conclusion, we have demonstrated that MALAT-1 is up-regulated in both human and mouse salivary gland pathologies and that both these diseases are associated with T antigen oncoprotein expression. We also show that BKV LT and ST antigens can up-regulate MALAT-1 in vitro in two different cell types (salivary/kidney) either by

direct infection or over-expression of protein antigens. Lastly, we have shown that BKV infection can regulate MALAT-1 at the transcription level. Because of its strong disease association, it is critical to understand mechanisms by which MALAT-1 expression is enhanced. Its modulation by the small DNA tumor virus BKV, is intriguing and opens the door toward understanding the regulation of this important malignancy associated gene.

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CHAPTER 5

BK VIRUS HAS TROPISM FOR HUMAN SALIVARY GLAND CELLS IN-VITRO: IMPLICATIONS FOR TRANSMISSION

The data from this chapter is reprinted with modifications from a published manuscript in Virology “LK Jeffers, V Madden and J Webster-Cyriaque, “BK virus has tropism for human salivary gland cells in vitro: Implications for transmission”

ABSTRACT

In this study, it was determined that BKV is shed in saliva and an in-vitro model system was developed whereby BKV can productively infect both submandibular (HSG) and parotid (HSY) salivary gland cell lines. BKV was detected in oral fluids using quantitative real-time PCR (QRT-PCR). BKV infection was determined using quantitative RT-PCR, immunofluorescence and immunoblotting assays. The infectivity of BKV was inhibited by pre-incubation of the virus with gangliosides that saturated the major capsid protein, VP1, halting receptor mediated BKV entry into salivary gland cells. Examination of infected cultures by transmission electron microscopy revealed 45-50 nm BK virions clearly visible within the cells. Subsequent to infection, encapsidated BK virus was detected in the supernatant. We thus demonstrated that BKV was detected in oral fluids and that BK infection and replication occur in-vitro in salivary gland cells. These data collectively suggest the potential for BKV oral route of transmission and oral pathogenesis.

INTRODUCTION

BK virus (BKV) belongs to the polyomavirus family and is ubiquitous in the human population [1]. The viral capsid is icosahedral and has a diameter of 45-50 nm. The genome consists of a closed circular double-stranded DNA molecule with approximately 5 kb. BKV genome consists of an early region, a late region and a regulatory region (RR) containing promoters, enhancers and the replication origin. The genome is transcribed bi-directionally from the origin, with the early region encoding for two non-structural proteins: large tumor antigen (T Ag), and small tumor antigen (t Ag). The late region encodes for structural capsid proteins VP1, VP2 and VP3 and the non-structural agnoprotein [1]. BKV initially binds to its cell surface receptor, an N-linked glycoprotein or ganglioside GD1b or GT1b, all of which contain an $\alpha(2,3)$ -linked sialic acid residue [2-3] and is caveolae-mediated endocytosed and traffics to the nucleus via the endoplasmic reticulum [4]. Viral uncoating is believed to occur within tubulo-reticular structures at the nuclear periphery prior to nuclear entry [4-5]. Once in the cell nucleus, the uncoated chromosome is transcribed. Early gene transcription products result in T antigens that cause quiescent cells to re-enter the cell cycle and increase cellular growth and proliferation. In permissive host cells the T antigens, acting as regulatory proteins, direct the remaining events, resulting in a productive infection. The completion of the process consists of viral DNA replication and transcription of late genes for the production of the structural proteins (VP1, VP2 and VP3) that constitute the capsid [1, 4, 6-8]. Viral capsomeres assemble around the daughter chromosomes in the nucleus, to form stable viral particles. Viral egress is thought to occur via cell lysis, although virions have been detected within vesicles in the cytoplasm [5].

BKV infection typically occurs during childhood, without specific symptoms, followed by a state of non-replicative infection in various tissues, with the urogenital tract as the principal site [9]. In the setting of relative or absolute cell-mediated immunosuppression, dramatic increase in BK viral replication occurs, resulting in the lytic destruction of infected uroepithelial cells, which in turn induces the influx of inflammatory immune cells [10]. This destruction of kidney cells most often occur in 5–8% of kidney transplants resulting in organ loss in half of these cases and is termed BKV-associated nephropathy (BKVN) [4, 7, 11]. Rise in the incidence of Polyomavirus viruria and viremia have been detected in recipients of bone marrow, kidney and heart transplants, as well as an increase in viruria in HIV-infected individuals [12]. The potential for BK replication at distant sites such as the salivary gland may certainly exist in the setting of HIV infection [12-13].

While it is clear that BKV infection is a ubiquitous childhood infection, BKV transmission is not currently well understood. The present studies were undertaken to determine whether BKV could infect and replicate within salivary gland cells resulting in virus production and potentially transmission. We have detected BKV in the saliva of patients with HIV-associated salivary gland pathology and in healthy individuals. In order to begin to decipher BKV pathogenesis within the salivary gland cell it was essential to develop an *in-vitro* model system. We have chosen to characterize BKV infection in submandibular (HSG) and parotid (HSY) gland cell lines. These cells were able to support viral entry, transcription, translation and virion production and BKV infection could be inhibited by saturating the capsid protein with its ganglioside receptor.

MATERIAL AND METHODS

Patients and Sample Collection

HIV positive patients from UNC hospitals dental clinic/infectious disease clinic and healthy volunteers were recruited to participate in the IRB approved study (04-DENT-356). Five milliliter saliva samples from HIV positive and negative individuals were collected, contents centrifuged and pelleted and DNA extracted by Qiagen DNeasy kit according to manufacturers' instruction.

Cell culture and virus

HSY cells are a neoplastic epithelial cell line initially established in culture from a human parotid gland adenocarcinoma[14]. HSG cells are an epithelial cell line isolated by using tissue culture techniques from an irradiated human submandibular salivary gland which showed no neoplastic lesion [15]. HSG and HSY cells were obtained as a gift from Dr. B. Baum (NIH) and cultured in McCoy's 5A medium (Sigma) and Dulbecco's minimal essential medium (DMEM; Sigma), respectively. African monkey kidney cells or Vero cells (American Type Culture Collection [ATCC]) were also cultured in DMEM (Sigma). All cell types were grown in medium supplemented with 10% fetal bovine serum (FBS) (Sigma), and 1% penicillin-streptomycin (pen/strep)(Gibco) unless otherwise stated and maintained in a humidified 37⁰C, 5%CO₂ chamber. BKV stocks were initially propagated in Vero cells from virus obtained from ATCC (VR-837). Viral lysates were made through three cycles of freezing the infected cells and supernatant at -80⁰C and thawing at 37⁰C.

Hemagglutination assay

BK virus was titrated using hemagglutination assay as previously described [16]. Briefly, 90 μ l of culture fluid was added to 10 μ l of 2.5% NP-40 in PBS and incubated at 37°C for 30 min. Two-fold dilutions of the treated-fluid in 25 μ l volume of HA buffer was transferred into V-bottomed 96-well microtiter plates and 25 μ l of a cold 0.5% suspension of guinea pig erythrocytes was added to each well. Plates were then incubated at 4°C for nonagglutinated cells to settle (about 2h). BK viral titer was taken to be the highest dilution in which >75% of the erythrocytes are agglutinated.

Conditions of infection

HSY, HSG and Vero cell monolayers were trypsinized, washed and resuspended in fresh culture medium supplemented with 2% FBS and 1% pen/strep and plated in poly-L Lysine coated 8-well chamber slides (Falcon), 12 well dishes or 35-mm diameter well culture plates at a concentration of 6.5×10^5 cells/ml. 64 HAU of BKV was then added to the dish and allowed to infect for 24h. At 24h post infection (hpi), virus was removed from the culture medium, cells were washed with 1X PBS and replaced with fresh medium. The infection of HSG and Vero cells was blocked by pre-incubation of BKV with 60 μ g/ml or 100 μ g/ml purified mixed gangliosides (Matreya, LLC) in media for 2-6 hrs at 37°C. At various times post infection the cell monolayers or supernatant were further processed for IFA, protein, RNA or DNA isolation, as described below.

Indirect immunofluorescence (IFA)

At stated times post-infection, cells were fixed for 10 min with 50% methanol/50% acetone, dried at RT then incubated at -20⁰C overnight for antigen retrieval. Fixed cells were then thawed at RT, rehydrated with 1XPBS/TritonX and incubated with PAb416 (Genetex) antibody (1:30) specific for SV40 T antigen. PAb416 has been shown to cross react with BKV T antigen and is commonly used for BKV T Ag detection. NCL-JCBK (Novocastra) antibody (1:20) was used to detect BKV VP1 protein. NCL-JCBK is specific for both BK and JC VP1 protein detection. Both antibodies were incubated with fixed cells for 1 hr at 37⁰C followed by a fluorescein-conjugated anti-mouse (Sigma) antibody (1:20). DAPI (Invitrogen) (1:10,000) was used to stain the nucleus and brefeldin A, BODIPY 558/568 conjugate to stain the endoplasmic reticulum (Invitrogen). At least ten random fields of positively stained cells were counted to determine percent infection. Nikon FXA with Q camera or Olympus IMT2 fluorescent microscopes were used to take photographs of cells.

Immunoblotting

Total cell protein was extracted using 1% SDS lysis buffer (1% (w/v) SDS, 0.05M Tris.Cl pH8, 1mM DTT). Protein concentrations were determined using the BioRad protein assay, and equal amounts of protein were electrophoresed on a 10% Bis-Tris polyacrylamide minigel (Invitrogen). PAb416 (1:200) (Genetex) in 5% non-fat dry milk in 0.1% Tween-20 PBS (PBS-T) was used to detect T Ag expression and Actin (C-11)-R sc-1615-R (1:1000)(Santa Cruz Biotechnology) in 1% BSA/TBS-T for actin expression. After washing in PBS/TBS-T, blots were probed with a horseradish

peroxidase-conjugated secondary antibody (1:10,000) (Promega). Antibody complexes were detected using SuperSignal West Pico Chemiluminescent substrate (Thermo scientific) and exposed to film (Kodak).

RNA isolation and real-time RT-PCR amplification

Total RNA was extracted using TRizol (Invitrogen) as described by the manufacturer. Contaminating DNAs were removed by use of RQ1 DNase kit (Promega) as described by the manufacturer. cDNA was generated using 20µg RNA, random primers and the SuperScriptTM II Reverse Transcriptase (RT) Kit (Invitrogen) as described by the manufacturer. A non-RT enzyme reaction was performed for each sample as a negative control for cDNA synthesis. cDNA was then subjected to real-time PCR analysis using Roche LightCycler 480 Syber Green I Master Mix as a detector in the Roche Light Cycler 480. Previously published primers for T Ag [17] and VP1 [18] were used. Gene expression values were normalized to the levels of β-actin transcripts, using the $2^{-\Delta\Delta C(T)}$ method, and are presented as the changes (*n*-fold) in T Ag and VP1 transcript levels, with the levels in uninfected/mock samples arbitrarily set to 1.

Encapsidated viral genome assay

The detection of infectious virus being released from HSG cells was performed on HSG cell supernatant collected at stated times post infection. Supernatant was passed through a 0.45µm filter and DNase-treated or used to infect Vero cells as previously described [3]. To degrade free DNA not encapsidated within virions, supernatant was treated with 250U DNase (Promega) or water for 15 min at 56⁰C, followed by enzyme

inactivation at 65°C for 10 min. To release viral DNA (vDNA) from capsids, proteinase K was used as described in the blood and body fluid spin protocol of the QIAamp DNA blood minikit (QIAGEN). DNA was eluted in 100 µl of sterile water. Levels of viral DNA were determined using primers for T Ag in real time PCR as described above. A plasmid, pBKV containing the entire genome of BKV, a gift from Volker Nicleit (UNC-CH) was used to derive standard curves for viral DNA quantitation.

Virus purification and labeling and confocal microscopy

BKV was propagated in Vero cells and purified as previously described[2]. Briefly, viral lysates, including all cell debris, were adjusted to pH 7.4 with 0.5 M HEPES (pH 8.0) and centrifuged at 8,000 x g for 30 min at 4°C. The supernatant was removed, and the pellet was resuspended in 5 ml of buffer A (10 mM HEPES [pH 7.9], 1 mM CaCl₂, 1 mM MgCl₂, and 5 mM KCl). The pH of the lysate was then adjusted to 6.0 with 0.5 M HEPES (pH 5.4), and 5 units of type V neuraminidase (Sigma) was added and incubated for 1 h at 25°C. The solution was returned to pH 7.4 through the addition of 0.5 M HEPES (pH 8.0) and then heated to 40°C and centrifuged at 16,000 x g for 5 min. The supernatant was removed and saved, while the pellet was further incubated with 5 ml of buffer A containing 0.1% deoxycholate at 25°C for 15 min and then centrifuged at 16,000 x g for 5 min. The supernatant was removed, combined with the previous supernatant, and centrifuged through a 15% sucrose cushion into 35 g/100 ml CsCl at 141,000 x g in a Beckman Optima LE-80K ultracentrifuge using a Beckman tube (28 by 89 mm) in a Beckman SW28 rotor for 3.5 h at 20°C. Virus was dialyzed against buffer A, and titers were determined using the hemmagglutination assay and DNase protection assay.

Purified BKV was then labeled using Alexa Fluor 488 microscale protein labeling kit (Molecular Probes) as directed by the manufacturer (bovine serum albumin (BSA) and 1X PBS were used as positive and negative labeling controls). Briefly, 100µl of purified viral solution or 1mg/ml BSA or 100µl 1X PBS was transferred to a reaction tube and 10µl of 1M sodium bicarbonate added, 33nmol Alexa Fluor tetrafluorophenyl ester was added and the reaction mixture was incubated for 15 min. After incubation, 50µl of the conjugate reaction mixture was layered on the resin bed in the microcentrifuge tube and centrifuged at 16,000xg for 1 min. The eluted virus was then used to infect HSG or Vero cells in poly-L lysine coated 8-well chamber slides (Falcon). At 24 and 48 hpi, monolayers of cells were fixed with 50% methanol/ 50% acetone for 10 minutes followed by addition of TO-PRO3 (Invitrogen) reagent at 1:10,000 for 2 min to stain the cell nucleus. Cells were then washed and stored in 1X PBS and used to detect virion entry using an Olympus FV 500 microscope.

Electron microscopy

HSG cells were infected with BKV or media (mock) and at 5 dpi the cells were washed with 1X PBS and the cell monolayers fixed with 3% glutaraldehyde in 0.15 M sodium phosphate buffer. Cells were then post-fixed with potassium ferrocyanide-reduced osmium tetroxide. Cells were embedded *in situ* in Polybed 812 epoxy resin and cut into ultrathin sections of 70 nm. Sections were post-stained in uranyl acetate and lead citrate then photographed using a LEO EM-910 transmission electron microscope (LEO Electron Microscopy Inc. Thornwood, NY) with Orius camera at 80 kV. All sections were cut parallel to the substrate.

RESULTS

BKV DNA detection in oral fluids.

Salivary shedding of BKV was assessed in a small cohort of healthy and immune suppressed individuals who had HIV disease. The mean BK copy number in saliva from individuals with HIV and an associated salivary gland disease was 3644 (n=11). HIV negative individuals had mean BK copy numbers that were significantly less at 26 (n=7) (Figure 1).

BK virions enter human salivary gland cells.

To determine whether BKV could enter salivary gland cells in-vitro virion labeling was employed (Figure 2). Fig. 2 shows CsCl purified BKV labeled with Alexa Fluor™ 488 (green) and the stained nucleus (red). Bovine serum albumin (BSA) and 1X PBS (data not shown) were also labeled with Alexa-Fluor™ 488 as positive and negative labeling controls, respectively. HSG or Vero cells were incubated with BKV at 64 HAU for 24 h, washed and fixed. Using confocal visualization, at 24 h BKV particles were located in the cell cytoplasm of both HSG and Vero cells. At 48 h BKV accumulation was detected at the perinuclear region of HSG cells. This type of perinuclear

accumulation has previously been described in human kidney cells in vivo [5] and in vitro [19]. We did not observe perinuclear accumulation of virions in Vero cells at the 48 h time point suggesting that viral uncoating and nuclear entry may have already occurred. It has previously been shown in human kidney cells that BKV traffics from the cell surface to the nucleus within 12 to 24 h [19] which may explain our inability to detect labeled BKV at the stated 48 h time point in Vero cells. The kinetics of entry and uncoating may be cell type specific.

BKV transcripts expressed in salivary gland cells.

Upon determination of BKV entry into salivary gland cells in vitro, the outcome of infection was assessed. Viral transcription levels of early gene, T Ag and the late gene, VP1 were examined. In both submandibular (HSG) and parotid gland (HSY) cell lines an increase in viral transcription of both T Ag and VP1 genes was observed up to 6 dpi (Figure 3). Beyond 6 dpi, cells became over-confluent, lifted off of the plate surface and began to die. A steady increase in transcription of both T Ag and VP1 transcripts was detected, generally peaking at 3 dpi. We consistently observed higher levels of VP1 versus T Ag transcripts in both cell types. This is consistent with the requirement for increased amounts of structural protein as compared to non-structural protein for viral replication to occur. Small amounts of T Ag are necessary to activate late gene transcription. Results were normalized to the levels of β -actin, using the $2^{-\Delta\Delta C(T)}$ method, and are presented as the changes (*n*-fold) in T Ag and VP1 transcript levels. Uninfected, mock samples were arbitrarily set to 1.

A.

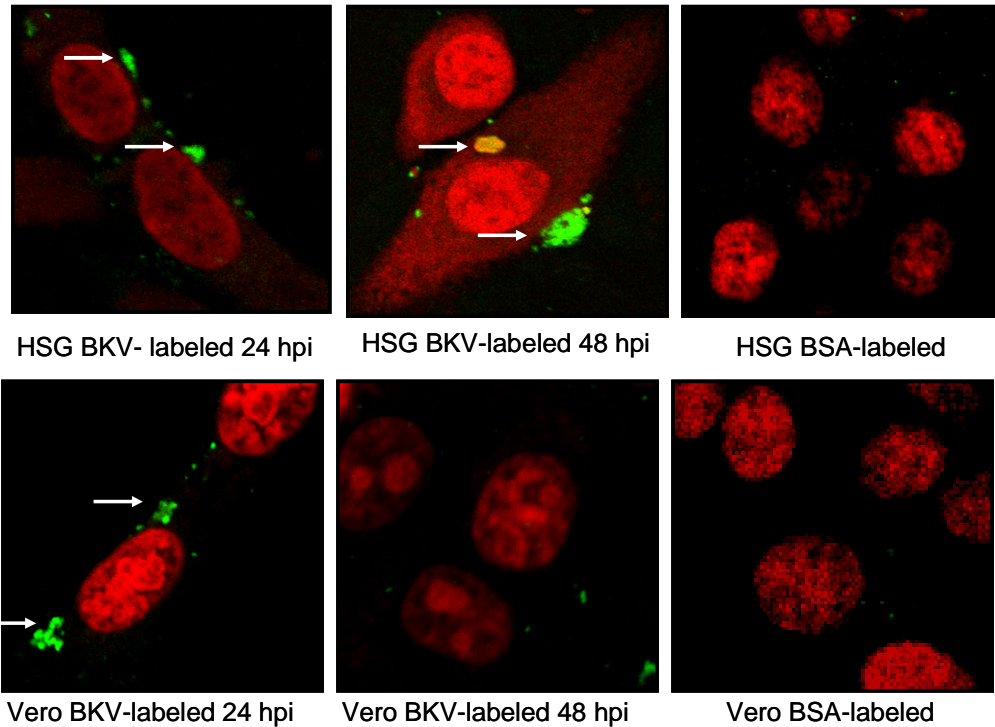


Figure 5.2 Detection of BK virions in salivary gland cell lines infected with BKV. A)

HSG and Vero cells were infected with Alexa-Fluor 488-labeled BK virus (green) or Alexa-Fluor 488-labeled BSA (green) or Alexa-Fluor 488-labeled 1X PBS (data not shown) as stated in the materials and methods. At stated times post infection, monolayers of cells were fixed and observed by confocal microscopy using a 63x lens objective. At 24 hpi, labeled BKV (green) was detected at the plasma cell membrane of HSG cells and within the cytoplasm of Vero cells. At 48 hpi, labeled BKV (green) was detected at the perinuclear region of HSG cells and not at all detected in Vero cells. Labeled BSA (green) and 1X PBS (data not shown) was detected only in the extracellular regions of both HSG and Vero cells. TO-PRO3 dye was used to stain the cell nuclei (red). White arrows point to Alexa-Fluor 488-labeled BK virions.

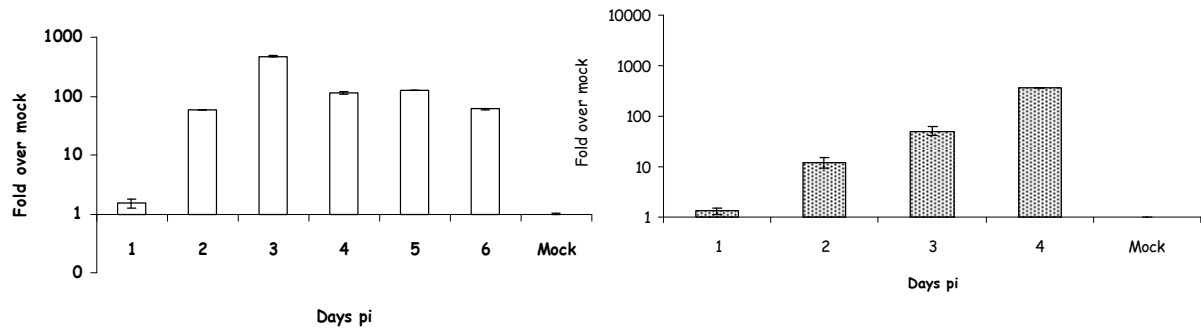
BKV protein expression in salivary gland cells.

Translation of T Ag in BKV-infected HSG cells was assessed by immunoblotting and immunofluorescence techniques (Figures 4A, 4B and 4C). As expected protein expression was delayed compared to mRNA transcription, consistent with previous work showing that BK virus replicates slowly in vitro [4, 8, 19]. Nuclear T Ag and VP1 protein expression was first detected at 4 dpi by IFA (Figure 4A and B). Based on counting of IF positive cells at 5 dpi (data not shown), an average of ten experiments determined the percentage of VP1 positive cells was approximately 15% and the percentage of T ag positive cells were 10% or less. Immunoblot consistently detected expression of both small and large T antigen at 4 dpi, although in one infection small amounts of protein were detected at day 3 (Figure 4C top and middle panel). Collectively, these data indicate that BKV gene expression occurred in human salivary gland cells in vitro.

Salivary gland cells release infectious virus into the supernatant.

In order to determine whether BKV infection of salivary gland cells results in productive infection, release of infectious virions from salivary gland cells was assessed. BK virions were not detected by electron microscopy in salivary gland cells days 2-4 dpi. However, at 5 dpi 45 -50 nm BK virions were identified within the cell cytoplasm similar to those virions detected within BKV infected kidney cells (decoy cells) shed into the urine of a kidney transplant patient diagnosed with BKV nephropathy (Figure 5). No viral particles were detected prior to 5 dpi within the cell cytoplasm.

T Ag



VP1

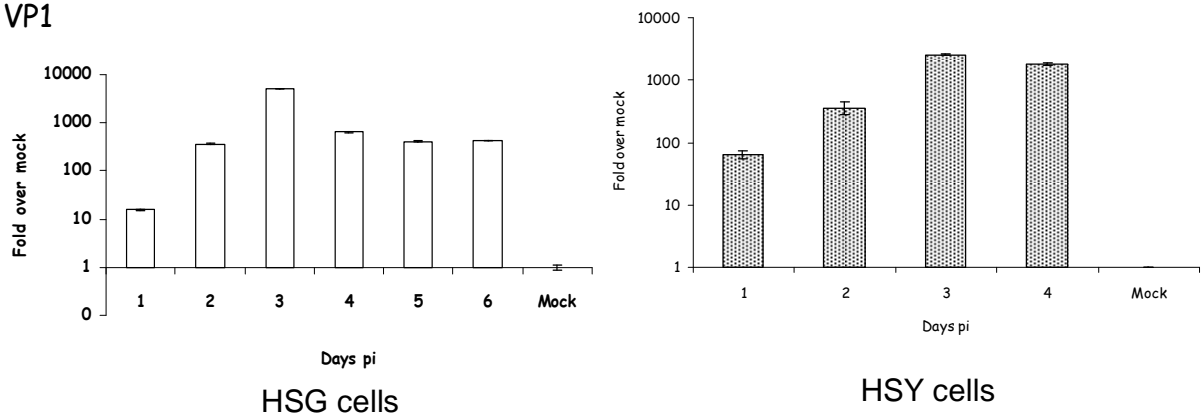
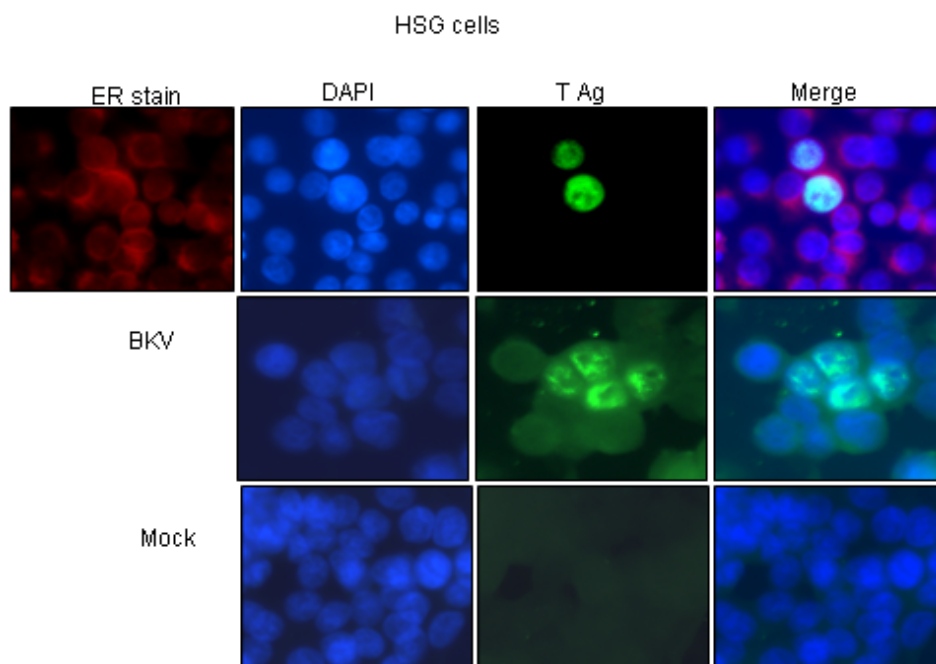
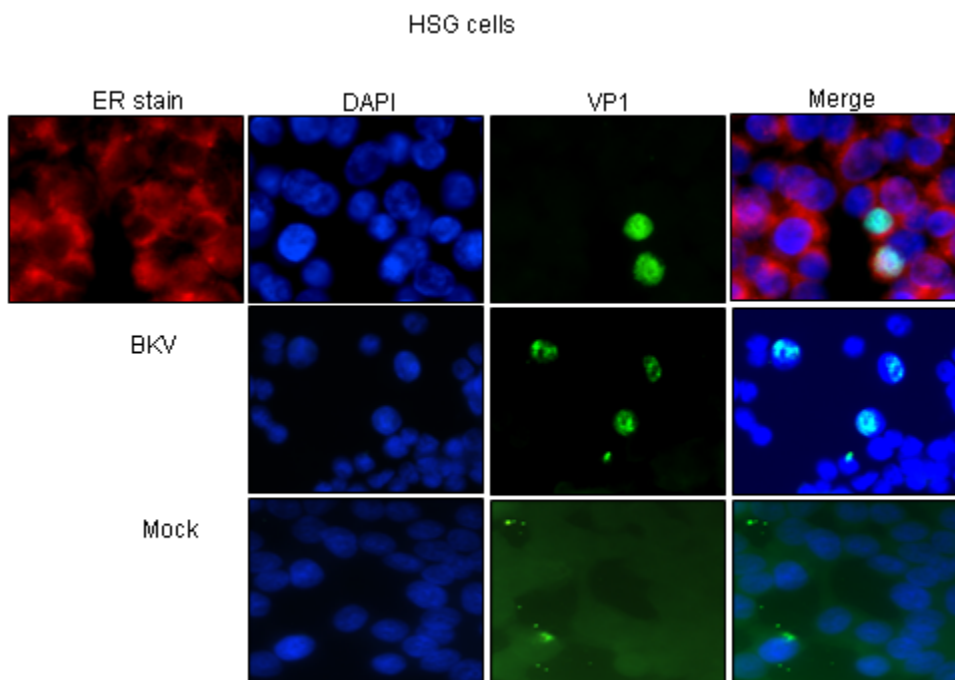


Figure 5.3. BKV replicates in human salivary gland cells. HSG and HSY cells were infected with BKV or media alone (mock) as described in the materials and methods. (A) At stated times post infection cells were collected, RNA isolated, cDNA generated and quantitative real time PCR performed for T Ag and VP1 viral transcripts. Gene expression values were normalized to the levels of β -actin transcripts, using the $2^{-\Delta\Delta C(T)}$ method, and are presented as the changes (*n*-fold) in T Ag and VP1 transcript levels, with the levels in uninfected/mock samples arbitrarily set 1.

A.



B.



C.

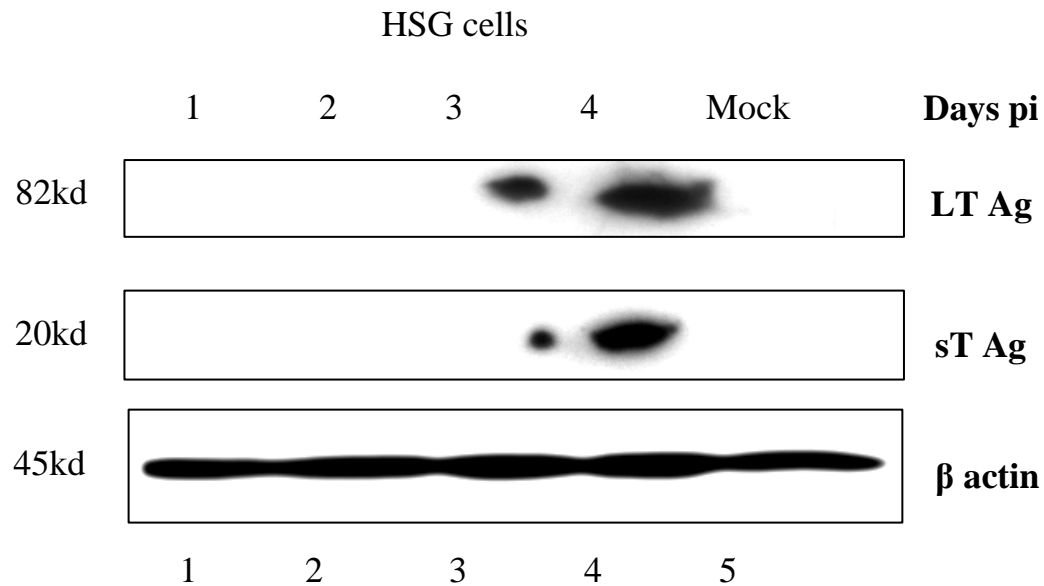


Figure 5.4 BKV protein expressed in salivary gland cells. At 5 dpi, monolayer of cells were fixed and probed for **A)** T Ag and **B)** VP1 expression using PAb 416 and NCL-JCBK antibodies, respectively. Photos are representative BKV-infected (top and middle panel) or control mock (bottom panel) HSG cells stained for T Ag (green) and VP1 (green). DAPI (blue) was used to stain the cell nuclei and Brefeldin A (red) used to stain the endoplasmic reticulum. (x20 magnification). **C)** At stated times post infection cell lysates were collected and used for immunoblotting as described in the materials and methods. Antibodies against T Ag (top panel) and β -actin (bottom panel) were used. T Ag protein was first detected at 4 dpi.

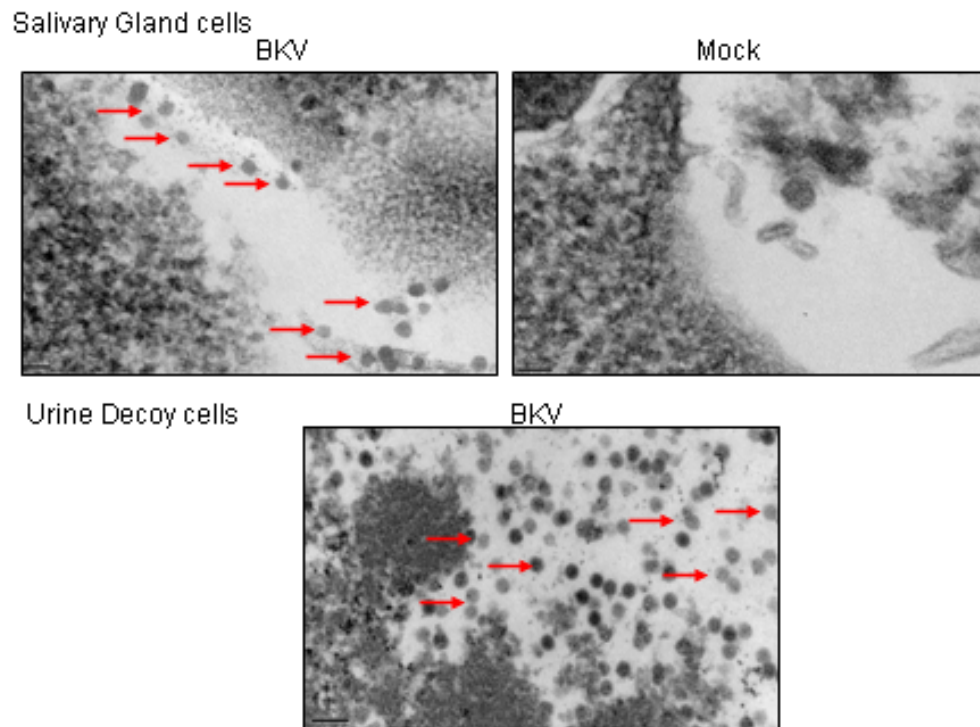


Figure 5.5 BK virions detected within salivary gland cells. Representative transmission electron micrograph (TEM) of BK virions at x160,000 magnification of BKV and mock-infected HSG cells (top panel) at 5 dpi. Bottom panel, BK virions from BKV infected kidney cells (decoy cells) in the urine of a BKV-associated nephritis patient

Time courses were performed during which supernatants from HSG cells were collected and filtered at 0, 1, 3, 4 and 5 days post-infection. Two separate experiments were performed in which 1) separate plates were infected and harvested at stated time points (data not shown) or 2) a single plate was infected from which supernatant was collected over time (Figure 6). Supernatants were then DNase/Proteinase K treated to detect only encapsidated viral DNA (vDNA) or Proteinase K-only treated as a control to detect both encapsidated and unencapsidated vDNA (data not shown), as previously described [3]. Over time post-infection increasing levels of virus was detected by quantitative real-time PCR indicating release of encapsidated viral DNA from infected cells.

The supernatants from HSG cells were also used to infect naive Vero cells. At 5 days post Vero cell treatment, cells were collected for quantitative real-time PCR, immunofluorescence, and immunoblotting. Again, real time results were normalized to the levels of β -actin transcripts using the $2^{-\Delta\Delta C(T)}$ method, and are presented as the changes (*n*-fold) in T Ag and VP1 transcript levels, with the levels in mock-treated samples arbitrarily set to 1. The level of T Ag and VP1 viral transcripts, were significantly higher in Vero cells treated with BKV-infected HSG supernatant over mock-infected HSG supernatant (Figure 7A). Immunofluorescence and immunoblotting techniques detected T Ag protein expressed in Vero cells treated with BKV-infected HSG supernatant (Figures 7 B and C). These data suggest that human salivary gland cells can indeed be productively infected with BK virus.

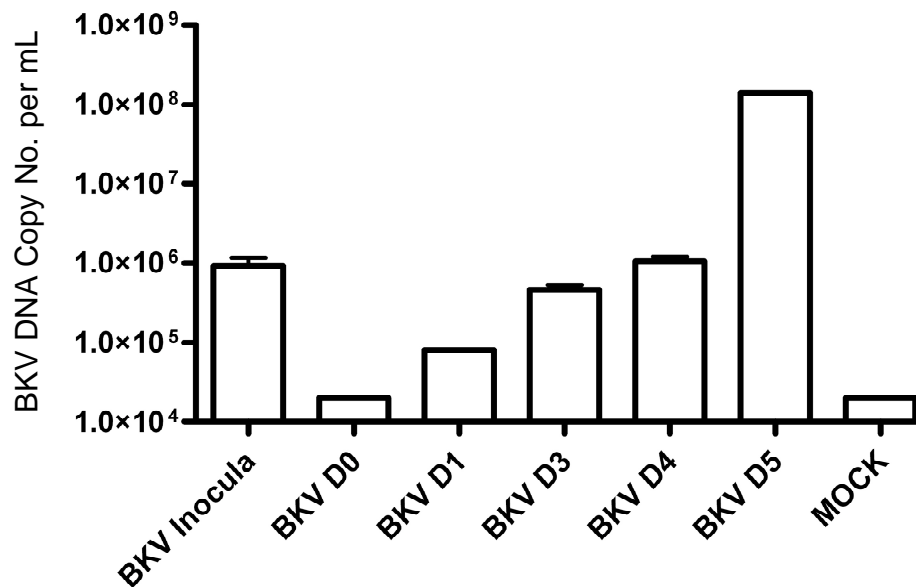
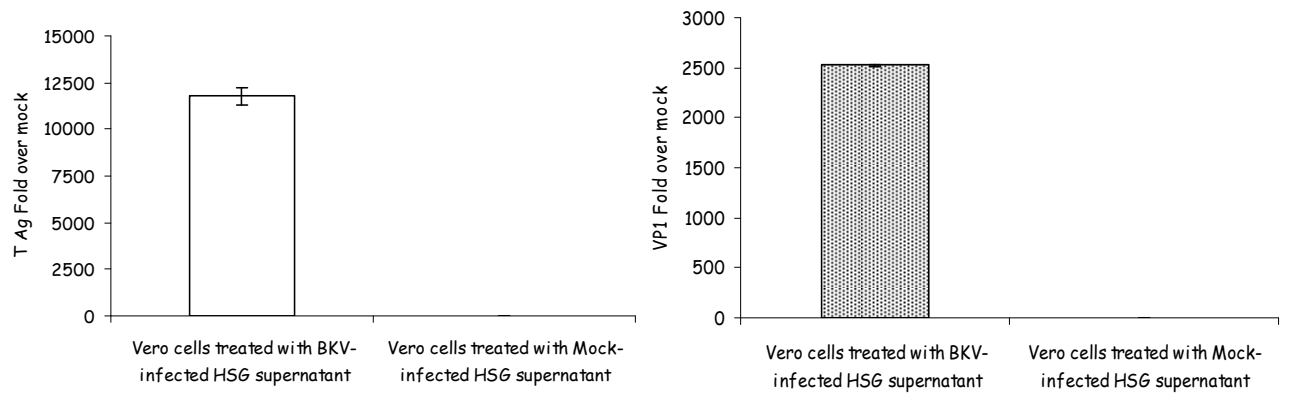


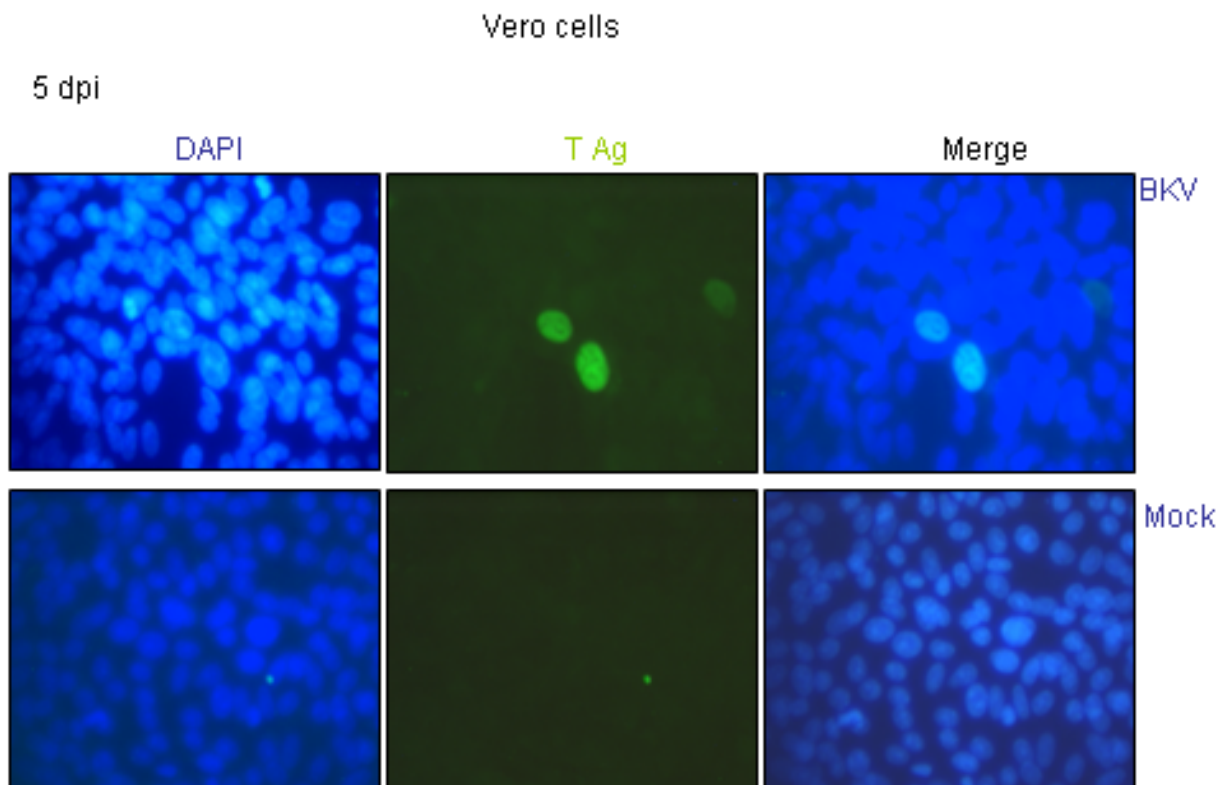
Figure 5.6.. Encapsidated BK virions released from human salivary gland cells.

Supernatant of BKV or mock (media only) infected HSG cells were collected at 0, 1, 3, 4 and 5 dpi, filtered and treated with either proteinase K only to quantify total vDNA (data not shown) or DNase followed by proteinase K treatment, to quantify encapsidated vDNA only, as described in the materials and methods. Eluted vDNA was then used to quantify T Ag copy number using real time PCR analysis. A standard curve (data not shown) was constructed using a plasmid coding for BKV T Ag to determine copy number.

A.



B.



C.

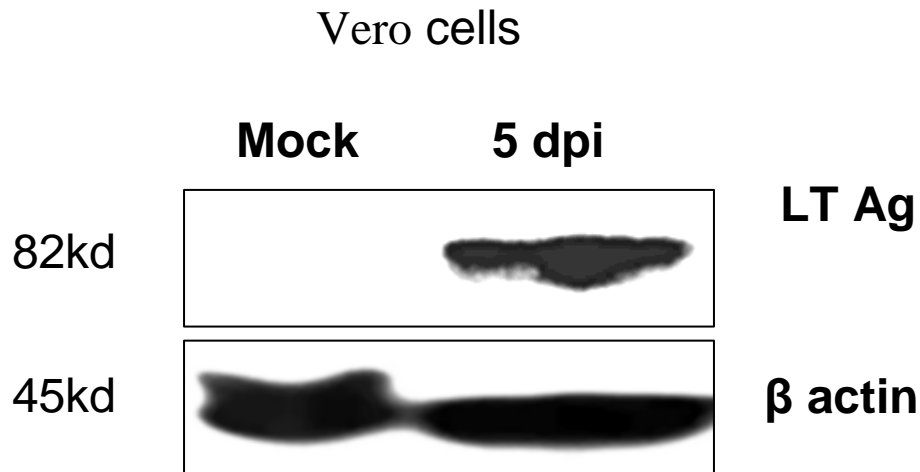


Figure 5.7. Infectious virus released from HSG cells. Supernatant of BKV or media only (mock) infected HSG cells were collected at 5 dpi, filtered and used to infect Vero cells as described in the materials and methods. At 5 days post treatment with HSG supernatant, Vero cells are examined for BKV infection by **A)** quantitative real time PCR analysis **B)** immunofluorescence and **C)** immunoblot. **A)** For real time PCR analysis, gene expression values were normalized to the levels of β -actin transcripts, using the $2^{-\Delta\Delta C(T)}$ method, and are presented as the changes (n -fold) in T Ag and VP1 transcript levels, with the levels in uninfected/mock samples arbitrarily set 1. **B)** Vero cells were fixed at day 5 pi and stained for T Ag (green). DAPI (blue) was used to stain the cell nuclei. Representative photograph of BKV T Ag and nuclear staining are shown for BKV-infected and control mock Vero cells. **C)** Immunoblot analysis of Vero cells for BKV T Ag (upper panel) and β -actin (lower panel) proteins detected at 5 days post HSG supernatant treatment.

BKV infects salivary gland cells via gangliosides.

It has previously been shown that BKV's receptors include an N-linked glycoprotein with $\alpha(2,3)$ -linked sialic acid and gangliosides, GD1b and GT1b [2-3, 20]. As HSG cells were permissive for BKV infection, the use of gangliosides for receptor-mediated BKV entry into these cells was assessed. A mixture containing all of these gangliosides in addition to GM1 and GD3 was utilized as the receptor for BKV on salivary gland cells was unknown. BKV was pre-incubated with GM1, GD1b, GT1b and GD3 to bind and saturate the capsid protein, VP1. Two different concentrations of ganglioside: 60ug/ml and 100ug/ml, were pre-incubated with BK virus prior to infection of HSG and Vero cells. Using real-time quantitative PCR, a 28 fold decrease was detected in viral transcription of T Ag in HSG cells infected with both BKV/60ug and BKV/100ug ganglioside mix compared to cells infected with BKV/DMSO alone. A 273 fold and 72 fold decrease was detected in Vero cells infected with BKV/60ug and BKV/100ug, respectively (Figure 8A). For VP1 viral transcripts in HSG cells we observed a 46 and 81 fold decrease in BKV/60ug and BKV/100ug ganglioside mix, respectively, compared to cells infected with BKV/DMSO alone. In Vero cells we detected a 402 and 62 fold decrease in VP1 transcripts when infected with BKV/60ug and BKV/100ug ganglioside mix, respectively.

A decrease in the number of T Ag expressing cells was also detected by IFA upon infection with the BKV/ganglioside mix versus cells exposed to BKV/DMSO (Figure 8B). In HSG cells, at 4 dpi, a 75% decrease in T Ag expressing cells was detected with the BKV/100ug ganglioside infection and a 45% decrease detected with BKV/60ug

ganglioside infection. While at 6 dpi, we observed 61% and 96% percent decrease in BKV/60ug and BKV/100ug infections, respectively. In Vero cells, at 4 and 6 dpi, a 100% decrease was detected with both the BKV/60ug and BKV/100ug ganglioside infections. These data indicate that human salivary gland cells are infected with BKV via receptor mediated VP1 entry and suggest that there may be a difference in receptor abundance between salivary gland cells and kidney cells.

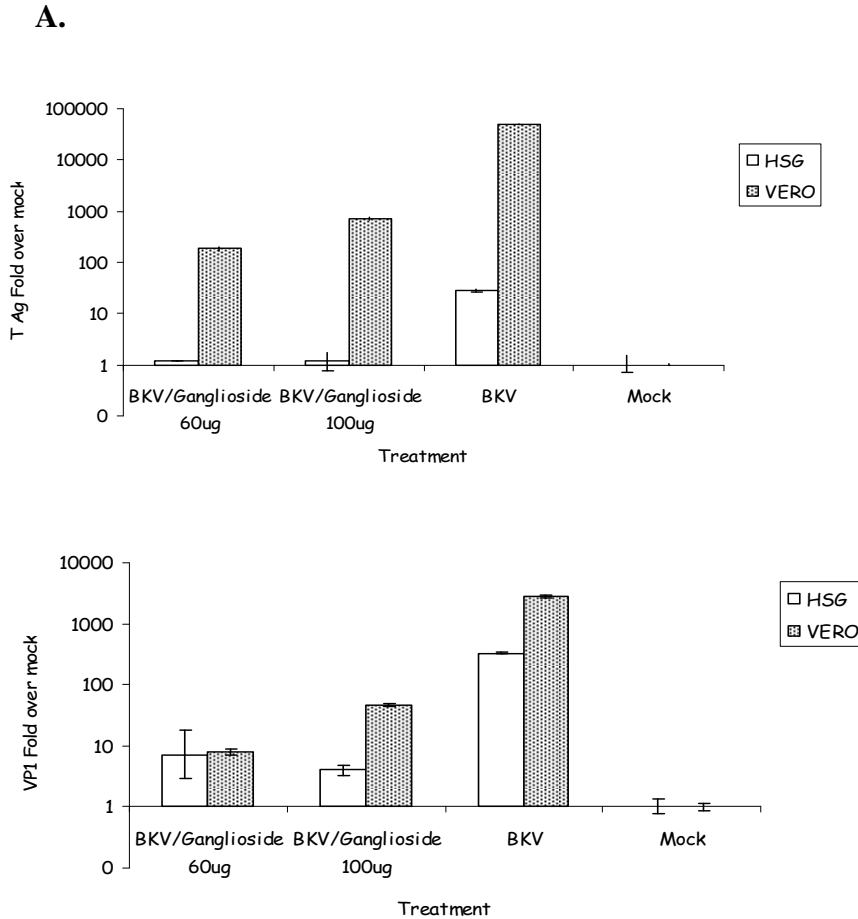


Figure 5.8 Inhibition of BKV infection in salivary gland cell lines via gangliosides.

HSG and Vero cells were infected with BKV in the absence or presence of a ganglioside mix or negative control media only (mock) and analyzed for T Ag expression at stated times post infection. **A)** Real time RT-PCR analysis of T Ag (top panel) and VP1 (bottom panel) transcripts from HSG (white bar) and Vero (checkered bar) cells at 5 dpi. Two concentrations of ganglioside mixtures were used, 60ug/ml or 100ug/ml, to incubate with BKV prior to infection. Gene expression values were normalized to the levels of β -actin transcripts, using the $2^{-\Delta\Delta C(T)}$ method, and are presented as the changes (*n*-fold) in T Ag/VP1 transcript levels, with the levels in uninfected/mock samples arbitrarily set to 1. Shown is a representative experiment, this study was performed three times in duplicate.

B.

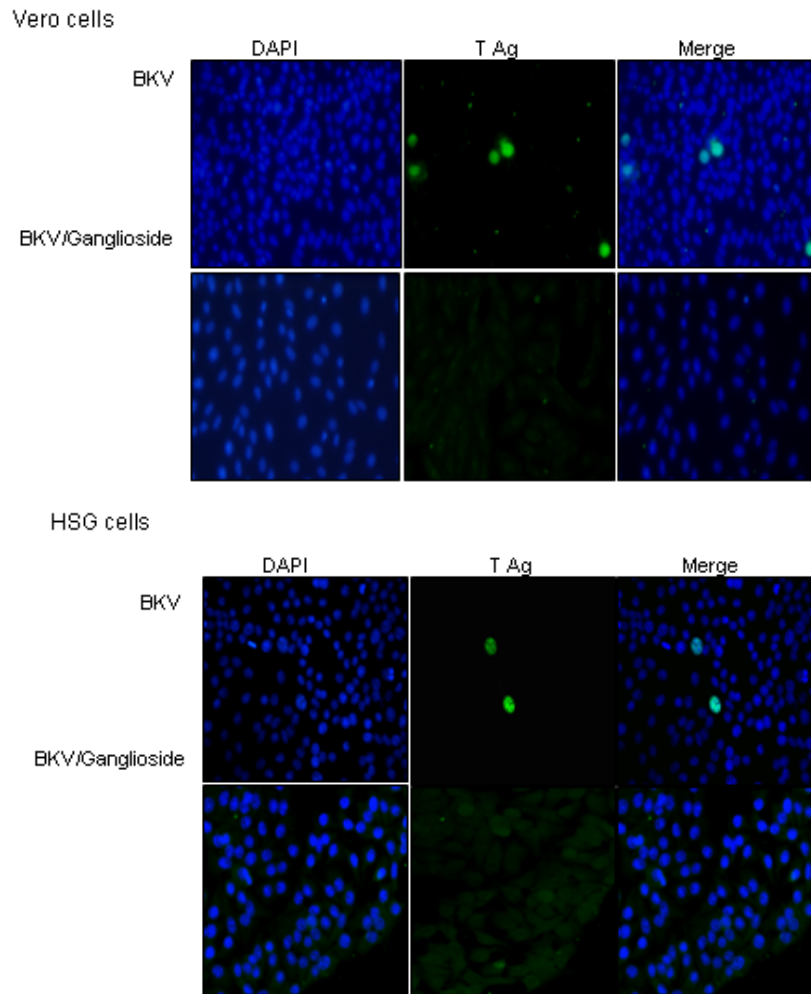


Figure 5.8 (continued). **B)** Immunofluorescence analysis of T Ag protein expression from HSG (top panel) and Vero (bottom panel) cells. At 4 and 6 dpi monolayer of cells were fixed and stained for T Ag (green) and DAPI (blue) used to stain the cell nuclei. The number of T Ag positive cells were counted per well and plotted as the percent decrease of T Ag positive cells in the presence of ganglioside compared to T Ag positive cells in the absence of ganglioside $[(100 - (\text{BKV only} \div \text{BKV/ganglioside} \times 100)]$. Bottom panel shows representative photos of IFA staining for T Ag in HSG and Vero cells. (x20 magnification).

DISCUSSION

The present data demonstrate for the first time, BKV detection in saliva and evidence that human salivary gland cells can be productively infected with BK virus. This suggests that while kidney/uroepithelial cells have long been known to be a site of BKV replication and latency, the salivary gland may also constitute an infectious reservoir for BKV. Although the natural route of BKV transmission has not been resolved [1, 9], our studies suggest a potential for oral BKV transmission.

Previous studies have investigated the presence of BKV DNA by polymerase chain reaction (PCR) in saliva from HIV-immunodeficient individuals and healthy controls but were not detected in their cohort [21]. Our studies however, using quantitative real-time PCR analysis detected BKV DNA in saliva samples from HIV positive patients and healthy controls. And like most opportunistic pathogens, BKV DNA was detected in the saliva at higher levels in immunocompromised patients compared to healthy individuals.

The detection of labeled BK virions within the cytoplasm of salivary gland cells confirmed BKV entry. In salivary gland cells, labeled virus was still detected at 48 hpi while labeled virus was no longer detected in Vero cells at the same time point perhaps due to viral uncoating and entry into the nucleus. Viral gene products were first observed at 24 hpi for both T Ag and VP1 using real time RT-PCR, with viral transcription peaking at about 3 dpi for both submandibular and parotid gland cell infection. Protein expression was first detected at 4 dpi by immunoblot and IFA. While BKV transcripts were detected in abundance the expression of viral encoded proteins were more modest. The difference in mRNA versus protein levels may reflect the decreased stability of the

BKV T Ag protein. Productive infection was further substantiated by the detection of virions by EM at 5 days post infection, as well as the presence of DNase resistant virus in the supernatant. These virions increased over time and were used to infect naïve Vero cells. Interestingly, the kinetics of infection appeared to be slower in salivary gland cells than in kidney cells.

BKV entry into salivary gland cells was inhibited by blocking VP1 via ganglioside saturation, as has previously been shown in Vero cells [22]. As expected viral transcription was more significantly diminished in the presence of 100 ug/ml ganglioside compared to 60 ug/ml in the salivary gland cells. Similar trends were observed in the Vero cell line upon ganglioside treatment. Likewise a consistent decrease in viral protein expression was detected with ganglioside treatment in both salivary gland and Vero cells.

The high incidence of BKV infection in the world population raises obvious questions about the mode of transmission from one individual to another. Given the known latency of the virus in the kidney, urine would appear to be a natural vehicle for spread within and between families. However, a variety of laboratory techniques have been used to assess the prevalence of viruria in the pediatric age groups, resulting in very low yield varying from 4% to 26.7% [23]. Transplacental transmission of BKV from mother to fetus has also been evaluated [24-27]. The data thus far however is controversial, with one group demonstrating 50% of their infants expressing BKV specific IgM antibodies in infants whose mothers seroconverted during pregnancy [25-26]. While others have shown consistently negative results for BKV-specific IgM in neonatal and cord blood samples drawn from the offspring of mothers excreting viral inclusion bearing cells in the urine [24]. Transplacental transmission of polyomavirus

has been demonstrated in mice [28], and it is conceivable that the same could occur in man. BKV DNA has also been detected in feces, blood, semen, genital tissues, peripheral blood mononuclear cells (PBMC), and normal skin biopsies, hence it is possible that the virus maybe transmitted by intimate contact with infected individuals [29-31].

The possibility of feco-oral transmission has recently been raised upon the detection of BKV DNA in urban sewage [29]. This possibility may also be considered as potentially complementary to our studies because swallowed material passes the mouth and nasopharynx and viruses transmitted through the oral-fecal route may be able to multiply in the throat, for example, in the salivary glands or tonsils. Additionally, both BKV and SV40 have been detected in the tonsils of immunocompetent children [32] and BKV detected in the nasopharyngeal aspirates of children with acute respiratory tract disease [21, 32-33]. Again, consistent with our studies indicating that BKV is present in the oral cavity and is possibly a mode of transmission from human to human. The presence of BKV in the saliva and its ability to replicate in salivary glands cells in vitro further suggests a role for oral transmission since BKV was detected in the saliva of both healthy and diseased patients in our study.

In conclusion, we have detected BKV in oral fluids and have shown that BKV is capable of permissive infection of salivary gland cells in vitro. To our knowledge these studies provide the first analysis of BKV salivary gland infection and replication. To the extent that parotid (HSY) and submandibular gland (HSG) cells can sustain low levels of BKV replication, substantiates BKV's presence in oral fluids originating within these glandular cells and confirms the potential for BKV oral transmission. These data may provide a foundation critical for further understanding BKV infection and pathogenesis.

The experiments described in this paper define a system in which BKV lytic replication can be studied in-vitro using a physiologically relevant cell type. The characterization of BKV infection of these cells set the groundwork for future studies to better understand how BKV is able to persist within the human host and reactivate under immunosuppressed conditions. Further studies will help us to identify factors that influence the vulnerability of salivary gland epithelial cells to BKV infection and more fully define BKV's role in disease. Determination of the cellular pathways that are altered may allow us to target and implement appropriate interventions that would decrease the morbidity associated with BKV-associated salivary gland pathologies.

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CHAPTER 6

GENERAL CONCLUSION

Salivary gland diseases (SGD) are characterized as enlargement of the parotid gland and development of lymphoepithelial cysts and lymphoma and/or a complaint of xerostomia causing pain, dental caries, oral candidiasis, difficulty eating and facial disfigurement. The goal of this dissertation was to investigate the role for an etiologic agent in salivary gland diseases, specifically HIV-associated salivary gland disease and Sjogren's Syndrome. HIV-SGD has been universally established as among the most important AIDS-associated oral lesions. In developing countries the incidence of HIV-SGD has been reported to be as high as 48% of HIV-1 pediatric patients and is increasing in the adult population worldwide [1-2]. SS on the other hand, is the second most common autoimmune disorder, affecting approximately four million Americans, with the disease affecting women 9 times more frequently than men. Histologically, HIV-SGD and SS are very similar except that in HIV-SGD, there is a lymphocytic infiltration of CD8 T cells compared to CD4 T cells in SS. Despite the fact that HIV-SGD and SS were both initially described twenty and two hundred years ago, respectively, the etiologic agent responsible for these diseases are currently unknown.

Epidemiologic data showing different rates of HIV-SGD in children (20-47%) versus adults (3-7.8%) suggest an opportunist as an etiologic agent [3]. Further, the incidence of salivary gland disease among HIV infected patients increased significantly following the introduction of HAART, reminiscent of opportunistic infections triggered by immune reconstitution [2]. Based on epidemiologic correlates and the link to lymphoma development, [4] we postulated that HIV-SGD was a manifestation of DNA tumor virus infection.

Opportunistic DNA tumor viruses include herpesvirus, papillomavirus and polyomavirus families. Herpesvirus and polyomavirus have been associated with significant human disease in immune compromised individuals eg. KSHV in HIV/AIDS and JCV in kidney transplant patients. Papillomavirus have been associated with cervical cancer and warts. Additionally, mice infected with polyoma virus develop enlarged parotid glands and a percentage of the mice develop malignancy.

While herpes and papilloma viral gene products were not detected in HIV-SGD, polyomavirus nucleic acids and antigens were consistently detected (Chapter 2). Polyomaviruses, SV40 and JCV DNA were not detected, however BKV-specific primers consistently detected BKV in HIV-SGD. BKV belongs to the polyomavirus family, along with SV40 and JC virus and is ubiquitous in the human population [5]. The viral capsid is icosahedral and has a diameter of 45-50 nm. The genome consists of a closed circular double-stranded DNA molecule with approximately 5 kb. BKV genome consists of an early region, a late region and a regulatory region (RR) containing promoters, enhancers and the replication origin. The genome is transcribed bi-directionally from the origin, with the early region encoding for two non-structural proteins: large tumor antigen (LTag), and small tumor antigen (STag). The late region encodes for structural capsid proteins VP1, VP2 and VP3 and the non-structural agnoprotein [5].

BKV infection typically occurs during childhood, without specific symptoms, followed by a state of non-replicative infection in various tissues, with the urogenital tract as the principal site [6]. In the setting of relative or absolute cell-mediated immunosuppression, dramatic increase in BK viral replication occurs, resulting in the lytic destruction of infected uroepithelial cells, which in turn induces the influx of

inflammatory immune cells [7]. This destruction of kidney cells most often occur in 5–8% of kidney transplants resulting in organ loss in half of these cases and is termed BKV-associated nephropathy (BKVN) [8-10]. Typically in BKVN there is a rise in the incidence of Polyomavirus shedding in the urine and blood.

This observation of BKV viruria and viremia in BKVN led to the investigation of BKV shedding in the oral cavity and peripheral blood of HIV-SGD patients in whom we previously detected BKV within the salivary gland. BKV shedding was detected in oral fluids of diseased patients at significantly higher levels compared to healthy subjects. Chapter 2 of this dissertation described the first report of a BKV salivary gland tropism, BKV shedding in oral fluids and evidence for a firm link between BKV and HIV-SGD. The presence of BKV in the saliva and its ability to replicate in salivary glands cells *in vivo* suggest a role for oral transmission.

In the case of SS, several viruses have been investigated as playing a role in disease, for example, Epstein Barr virus (EBV), Cytomegalovirus (CMV) and Coxsackievirus but the evidence is contradictory and requires further study. In chapter 3 of this dissertation, preliminary studies indicate an association between SS and BKV, however more patient samples are needed to increase the statistical significance of causation between BKV and salivary gland pathology, including saliva, blood and urine samples. This is the first description however of an association between BKV infection and SS.

To understand the consequence of BKV infection in HIV-SGD, differential gene expression studies were performed and was described in chapter four of this dissertation. Suppressive subtractive hybridization (SSH) technique was used to compare gene

expression profiles between HIV-SGD and healthy control biopsies. The results indicated that a novel non coding RNA, metastasis associated lung adenocarcinoma transcript 1 (MALAT-1) was highly up-regulated in the disease. Typically MALAT-1 is widely expressed in normal tissue and while little is known about its function, appears to be a potentially generic marker for epithelial carcinomas [11-16].

MALAT-1 up-regulation was consistently detected in BKV T antigen-expressing HIV-SGD. A mouse model of polyomavirus associated salivary gland disease was explored to confirm Tag mediated MALAT-1 up-regulation in vivo. Mice infected with polyomaviruses not only develop salivary gland disease but often go on to develop malignancies within the glands [17-18]. A mouse polyoma middle T antigen-expressing transgenic model with SGD pathology was used [17]. Interestingly, MALAT-1 was highly expressed in transgenic animals expressing the functional homologue of the BKV oncoprotein compared to wild type animals. Similarly in SS, MALAT-1 was determined to be up-regulated in patients in whom BKV was detected. This dissertation describes for the first time the association of a DNA tumor virus with increased expression of a tumor-associated transcript within two similar disorders (HIV-SGD and SS), both of which have been shown to be occasionally associated with lymphoma development.

In vitro studies were also performed to confirm BKV's role in MALAT-1 up-regulation. Cell lines expressing BKV gene products also resulted in over-expression of the MALAT-1 transcript as shown in chapter 4 of this dissertation. In an initial step toward understanding how BKV regulates MALAT-1, a reporter assay was developed to assay activation of MALAT-1's enhancer/promoter region. Upon BKV infection increased reporter activity was observed suggesting that BKV can regulate MALAT-1 at

the transcription level. This is the first description of a mechanism for MALAT-1 regulation.

Closer inspection of the MALAT-1 gene revealed features of a primary miRNA (pri-miRNA) at its 3' end. miRNAs are single-stranded RNA molecules of 21-23 nucleotides in length, which regulate gene expression. miRNAs are encoded by genes from whose DNA they are transcribed but miRNAs are not translated into protein (i.e. they are non-coding RNAs); instead each primary transcript (a pri-miRNA) is processed into a short stem-loop structure called a pre-miRNA and finally into a functional miRNA. Mature miRNA molecules are partially complementary to one or more messenger RNA (mRNA) molecules, and their main function is to down-regulate gene expression. Like known miRNAs, MALAT-1 is a non-coding transcript, folds into a short stem loop structure at the 3' end and is conserved across mammalian species. Investigation of MALAT-1 as a miRNA is currently being explored and potential targets in SGD will be determined.

All of the work on BKV pathogenesis to date has been performed in kidney cells, the known site of viral latency. To truly understand BKV pathogenesis in SGD and to confirm host cell gene modulations observed in HIV-SGD and SS, an in vitro model of BKV infection of salivary gland cells was created. Each step of BKVs viral life cycle was examined, including viral entry, replication, egress and infectivity. Parotid (HSY) and submandibular (HSG) salivary gland cell lines were used to create the in vitro model. Using these cells, we were able to observe viral entry, mRNA transcription, protein expression and DNA replication over the course of infection. Results indicated that BKV was capable of replicating efficiently and producing infectious virus particles within

these cells. We also determined that BKV entered these cells via gangliosides, as has previously been shown to be a receptor for BK virus on kidney cells. To our knowledge these studies provide the first analysis of BKV salivary gland infection and replication. To the extent that parotid (HSY) and submandibular gland (HSG) cells can sustain low levels of BKV replication, this substantiates BKV's presence in oral fluids originating within these glandular cells and confirms the potential for BKV oral transmission.

Further evidence confirming that BKV plays a role in HIV-SGD was published by Leggott et. Al [19]. Leggott described a pediatric case of HIV-SGD in which the patient had been diagnosed for more than 5 years, and had considerable disfiguring enlargement that was firm, painless and associated with xerostomia and rampant caries. This patient had two labial salivary gland biopsies, and a fine-needle biopsy of the parotid demonstrated no evidence of EBV, CMV or HIV in the tissues. The same child, after 5 years of chronic parotid swelling, started steroid therapy for her HIV-associated kidney disease. Within 4 days of starting therapy, her parotid enlargement had completely resolved. This suggests perhaps an inflammatory origin for the lesion or possibly a viral infection that may have been present both in the kidneys and parotid gland that was resolved simultaneously, such as the BK virus which causes kidney disease in immune suppressed individuals which we have shown to be present in salivary glands cells of HIV-SGD patients (Chapter 2).

Based on my work and findings in the literature, a model has been proposed to describe the pathology of BKV in an individual that develops salivary gland disease (Figure 6). BKV is initially acquired via the oral route and can be detected in the saliva and salivary glands. Previous work from different groups have detected BKV in the tonsil

and peripheral blood lymphocytes and may serve as a reservoir for BKV latency as well as a means for dissemination. BK virions can be transported from the salivary gland and tonsil through the blood stream via PBMCs to the kidney where it is known to reside and remain latent. During immune-suppression, for example in transplant recipients, autoimmune disease or in HIV positive individuals, BKV can then become active and replicate resulting in kidney disease and/or salivary gland disorders. The factors that contribute to BKV-induced disease are largely unknown. However, epidemiological studies suggest that the most significant cofactor leading to BKV-induced disease is the degree of immunosuppression [20-21]. Reactivated virus can then be detected in the urine and saliva and may be important in feco-oral transmission. Viral replication in the salivary gland would then lead to up-regulation of the tumor-associated transcript, MALAT-1 which has the potential to play a role in malignancy development.

Based on these studies, it is clear that BKV plays a role in salivary gland disorders by infecting and replicating in salivary gland cells. As a result of BKV replication within salivary gland cells in immune compromised individuals host cell gene modulations occur, as has been shown. Additional studies need to be performed however to further investigate the many genes de-regulated in HIV-SGD. Future work include characterization of the distinct role of lymphoid/inflammatory infiltrates and of salivary epithelial cells in the disease. Using the in vitro model, experiments to confirm differential gene expression analysis from HIV-SGD biopsies can be performed. In addition, development of an agent-based model that models viral and host protein interactions during BKV replication is being created in collaboration with Suzy Vasa and Dr. Morgan Giddings based on the in-vitro model. Collaborations are also currently

underway to extend the in vitro model to an ex vivo model whereby salivary gland cells in organotypic cultures, that is salivary gland cells explanted from human tissue that differentiate into three-dimensional salivary gland structures similar in function and structure to natural human salivary glands, will be infected with BKV and characterized.

Work from this dissertation will foster studies to better understand how BKV is able to persist within the human host and reactivate under immune suppressed conditions. Determination of the cellular pathways that are altered may allow us to target and implement appropriate interventions that would decrease the morbidity associated with HIV-SGD. Currently, Cidofovir is being tested on its effect in BKV-associated kidney disease, using our in vitro model we will be able to test the effectiveness of drug therapy on BKV replication and infection of salivary gland cells. The fact that both HIV-SGD and SS are associated with malignancies ties in the possibility that MALAT-1 up-regulation observed in these diseases is real and can be the causative agent for these lesions. Of course more work would be needed to confirm our preliminary observation and the potential for a new miRNA to be described is also very powerful and will be further investigated.

The ability for this work to translate into clinical studies is important seeing that translational research is an important factor in today's society. Drug testing and host and cellular gene expression studies can further add to our knowledge of these disease processes and allow us to progress our understanding of critical gaps in salivary gland disorders. The long range goal of this project is to define the interrelationship of viral infection with treatment and prevention strategies.

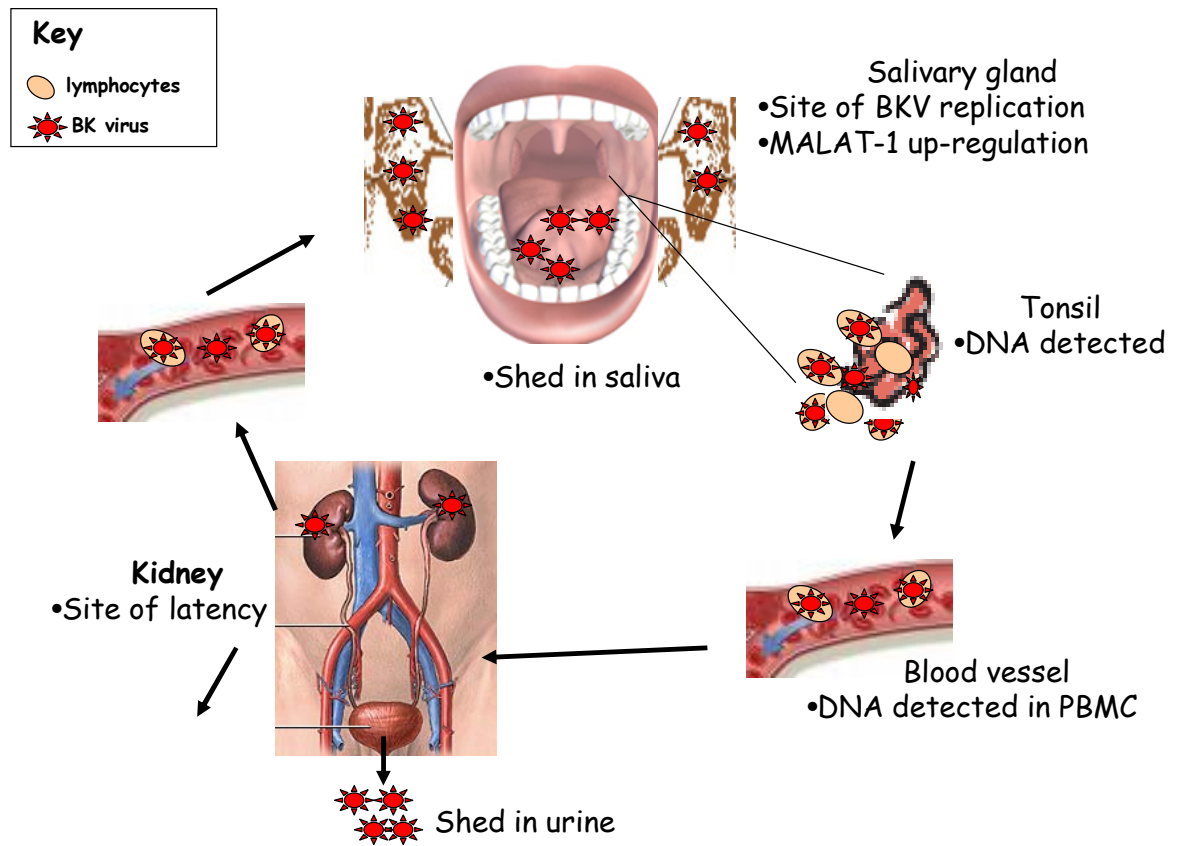


Figure 6. Proposed model of BKV pathogenesis in a patient with salivary gland disease .

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